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CLINICAL AND EXPERIMENTAL

DROP TEST STUDIES ON BLOOD: THEIR PROGNOSTIC VALUE IN THROMBOEMBOLISM

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WASHINGTON, D. C.

INTRODUCTION

THIS report will present the preliminary evaluation of a six-year study comprising of more than 10,000 observations on certain physicochemical blood variations in 1,000 individuals in an attempt to determine the usefulness of such variations as guides in the prediction and treatment of thromboembolism. The physicochemical variations observed were those reflected by a drop test that includes the combined effects of viscosity, surface tension, and specific gravity in one result called the stactometric ratio. It was presumed that these measurements would provide a quantitative index for the blood composition factor of Virchow's triad for thromboembolism (vascular damage, stasis of blood flow, and changes in blood composition) and also indicate the resistance to blood flow that might be due to increased viscosity. As the data accumulated, it became apparent that the variations were blood reflections of the changes in general physiologic activity to physical stress, and patterns soon emerged classifying individuals into normoreactor, hyporeactor and hyperreactor groups, each having diagnostic and prognostic significance.

METHODS AND MATERIALS

General Principle.—The stactometric ratio of the blood was determined by the drop test previously described[1] with some modifications and improvements in technique. Essentially, it consists in timing, under standard conditions of temperature and pressure, the interval taken by 14 drops to fall away from the tip of a standardized and sterilized,

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dry test instrument (stactometer) containing 1 ml. of blood drawn directly into it from a vein. The stactometer is standardized by timing its delivery of 14 drops from 1 ml. of 77° F. (25° C.) distilled water. The stactometric ratio is calculated by dividing the blood time by the water time. The delivery of the drop to the tip will depend upon the viscosity of the liquid and the pressure head in the stactometer, while the falling away of the drop will depend upon its surface tension and specific gravity. The test drops and the remainder of the milliliter of blood in the stactometer are caught in a heparinized test tube and used for a hematocrit determination. Since the rate of the blood dropping from the stactometer is influenced by the combined effects of the cellular and plasma elements, it was thought that a hematocrit determination on the same sample of blood might provide one explanation for some of the stactometric ratio fluctuations. It was found that there was an empirical relationship between the stactometric ratio and hematocrit figures, and the hematorcrit emerged as a convenient, built-in base line along which stactometric ratio fluctuations could be plotted in terms of plus or minus variations. This stactometric ratio to hematocrit relationship and its significance as it unfolded will be clarified in the discussion of the results.

In the original report,[1] the term viscosity index was used to denote the result that is now called the stactometric ratio. At that time it was thought that viscosity was the main function being tested because of the correlation between the normal figures obtained by the drop test and normal figures for blood viscosity obtained by other methods.[2] However, McCleery and co-workers,[3] referring to the original report,[1] were not satisfied with the prognostic significance of viscosity studies using a Hellige viscosimeter, which does not include the factors of surface tension and specific gravity in its result. Since there may be considerable differences in the results arrived at by the drop test and the results that might be obtained by other methods of measuring viscosity in the disease states studied, the term viscosity index has been replaced with stactometric (drop measuring) ratio in this report.

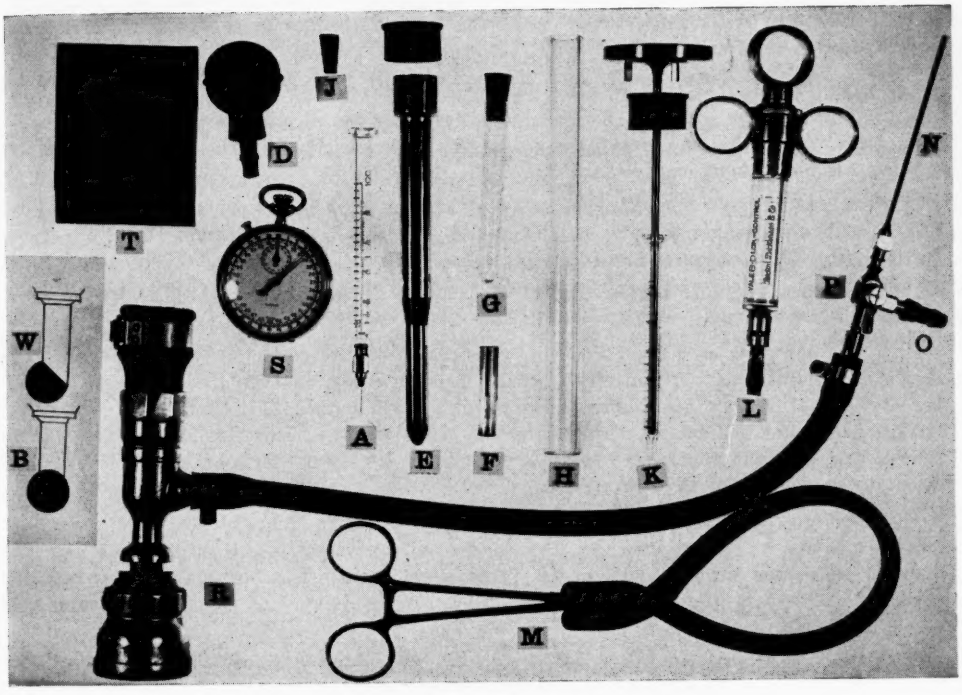
Equipment and Materials.—The particular equipment and materials used in this study, illustrated and identified by the capital letters in Fig. 1, consist of the following:

1. The stactometer (*A*) is made up of a 1 ml. B-D Glasbestos syringe barrel 1GTC* with a 25 gauge ½ inch VIM hypodermic needle attached. A group of 8 stactometers was used in this project, and the syringe barrel for each was selected so that the distance from the tip of the glass nipple to the 1 ml. mark was the same (7.2 cm. in these stactometers), thus ensuring an equal column of liquid pressure in each stactometer. The needles used gave stactometers that delivered 14 drops of 77° F. distilled water in 11.5 to 12.5 seconds. A convenient way of identifying each stactometer is to scratch away the red color in the groove opposite the appropriate number in the scale on the syringe barrel. Each stactometer is stored in a 2 ml. B-D Steritube 300A* (*E*). A 3 by 0.5 cm. piece of aluminum tubing (*F*) is placed in the bottom of each Steritube to guard against the needle's dropping off and damaging its tip during autoclaving. The fluid to be tested is drawn into the stactometer through the needle by use of suction from a ¼ oz. bulb (*D*) removed from a B-D Asepto Syringe 2040* and fitted with a B-D adapter 1/606* shod with 1.5 cm. of ⅛" rubber tubing. This adapter fits snugly into the glass, flanged end (hereafter referred to as the suction end) of the stactometer, and can readily be removed after it has drawn the fluid up to the 1 ml. mark. Each stactometer is provided with a small cork (*J*) to fit in the suction end; the use of this cork will be mentioned in the section on cleaning the stactometer.

2. Test tubes (*G*) 7.5 by 0.75 cm. containing 1 drop of 50 mg. per milliliter heparin and allowed to dry. One tube with cork is necessary for each test.

3. A test stand consisting of a 17.5 by 1 cm. glass tube (*H*). This stand is placed upright in a clip holder provided in a corner of the apparatus box (*T*), and a heparinized test tube (*G*) is dropped into it in preparation for a test. When the stactometer is placed into the test stand, the needle hub rests on the rim of the heparinized tube while the

*B-D Products Catalog Condensed, Jan., 1952. Becton, Dickinson & Co., Rutherford, N. J.



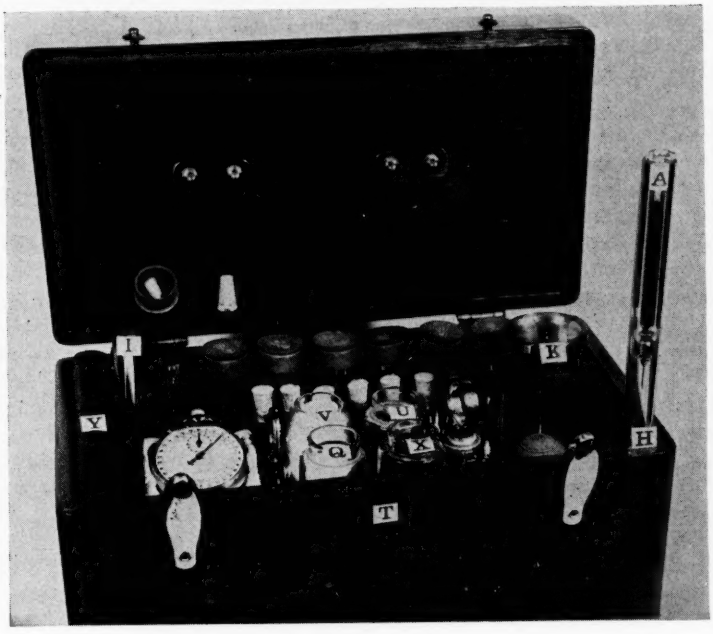


Fig. 1.

flange of the upper end rests on the upper rim of the test stand. Thus, the stactometer is held steady in a vertical position while the drops fall away in full view without interference from air currents.

4. A stop watch (*S*). This one records to the tenth of a second.

5. Four, 50 ml. capacity, 9 by 2 cm. glass vials for alcohol sponges (*V*), tap water (*Q*), waste water (*X*), and cleaning solution (*U*). The cleaning solution used was Alconox,* a blood dissolving, nonfilming preparation.

6. A washing syringe (*L*), consisting of a 5 ml. B-D Yale Luer Lok Control Syringe 5YLC† with an attached adapter similar to the one on the suction bulb. This syringe is for cleaning the stactometer immediately after completion of the test.

7. A tourniquet (*M*), consisting of a 35 cm. length of rubber tubing and a hemostat to provide easy control.

8. A thermometer (*K*) (Weston, ordinarily used for photographic solutions). The long stem of this dial thermometer is pushed through a hole made in the middle of the rubber cap of a Steritube containing a Glasbestos syringe barrel. The stem, thus, lies in the syringe barrel and indicates its temperature in the Steritube. This thermometer-fitted Steritube is placed in the apparatus box along with the stactometer Steritubes and serves to indicate the temperature of the stactometers.

9. An apparatus box (*T*) 18.5 by 14.5 by 27 cm. This box, provided with a carrying handle, holds in individual slots the above-mentioned equipment plus a record book (*Y*) and a small slide rule for calculating the stactometric ratio. The box makes it possible to perform the test at the bedside, and provides for the important immediate cleaning of the stactometer.

10. A Penberthy Aspirator (*R*), with rubber tubing and attached, three-way B-D Stop-Cock 1/S† (*P*), whose adapter arm (*O*) is shod with 1.5 cm. of ⅛" rubber tubing and the other arm fitted with a 15 gauge, 9 cm. needle (*N*) whose beveled tip is filed off. This aspirator will fit most faucets to provide water under pressure through the 15 gauge needle for washing the inside of the stactometer, and it will provide suction through the adapter arm (*O*) which can be placed in the suction end of the stactometer to aspirate distilled water for rinsing and air for drying.

Procedure.—The stactometer temperature is brought to 80° ± 2° F. before standardizing or testing blood. At ordinary room temperature of about 77° F., the usual handling of the stactometer will warm it to the desired range of temperature. Quick warming of a cold (65° to 75° F.) stactometer may be accomplished by gripping its barrel for about 1 minute. Cooling may be readily produced by wiping the stactometer barrel with an alcohol sponge and allowing evaporation for about 1 minute; this will reduce the temperature from 90° to 80° F.

Standardization of the stactometer is accomplished by drawing 77° F. distilled water up to the 1 ml. mark and timing the interval between the fall of the first and fifteenth drop from the tip of the needle. The drops of water falling from the needle tip should leave from the bevel side as in *W* (Fig. 1), and not as in *B*, to obtain constant results; and they should drop cleanly without hesitating or twisting. The standardization figure assigned to each new stactometer is the average of 25 postautoclaving standardizations during routine use on normal individuals. This standardization figure, once established, need be confirmed only about once a month, and variations of plus or minus 0.75 second are acceptable. However, to make sure that the stactometers are always within the allowable standardization range, a check standardization is done before each autoclaving. This check standardization is usually 0.3 to 0.5 second faster than the assigned standardization figure. The group of 8 stactometers used in this study had standardization figures of 11.5, 11.8, 11.8, 12.2, 12.3, and 12.5 seconds.

The blood to be tested may be drawn from any vein, but the venipuncture should be in an area not recently traumatized. The turniquet is applied only long enough to per-

*Alconox, Inc., New York, N. Y.

†See footnote page 500.

mit a good, quick venipuncture (15 to 30 seconds), and is released before blood is drawn into the stactometer. The blood is drawn to the 1 ml. mark, and the suction bulb removed before the needle is withdrawn from the vein; the needle is in the vein for about 25 seconds. The blood-laden stactometer is immediately placed in the prepared test stand and the stop watch started with the falling away of the first drop and stopped with the discharge of the fifteenth drop (the time is usually between 45 and 55 seconds for normal blood). The drops should fall away at regularly increasing intervals, and, except for the first drop or so, will encircle the whole tip of the needle as in *B* (Fig. 1). The remainder of the blood in the stactometer is ejected into the heparinized test tube and used for a hematocrit determination in a Wintrobe tube later.

Immediately upon completion of a blood test, the stactometer is cleaned by forcing tap water through it by means of the cleaning syringe (*L*) which is loaded from the convenient vial (*Q*). The stactometer needle is then immersed in the cleaning solution vial (*U*), and the cleanser aspirated into the stactometer barrel until it is full, whereupon it is stoppered with a small cork (*J*). Thus, the solution will remain suspended in the stactometer and dissolve any remaining blood, which will then be easily removed in the later, final cleaning.

The final cleaning consists in clearing the stactometer needle with a stylet and scrubbing the inside of the cleanser-containing glass barrel with a small brush. The needle tip is checked and any burring corrected. Warm tap water through the 15 gauge needle (*N*) of the Penberthy Aspirator is flushed through the stactometer to rinse it. While the water is pouring through the stactometer, its needle is temporarily disengaged and the water allowed to shoot from the glass nipple into the hollow hub, thus washing out any hidden particles.

After the stactometers have been washed and are still full of tap water, each is held up and the tip of its needle is wiped with a silicone-impregnated tissue paper (*T*)* until the drops of water fall away from the bevel side of the needle as in *W*. Each stactometer is finally rinsed by immersing its needle into a supply of distilled water and applying suction through the adapter arm (*O*) of the Penberthy Aspirator, and then drying it by allowing the suction to continue while the needle is exposed to the air. This is followed by check standardizing, again wiping the needle tip with silicone tissue and autoclaving. The stactometers are autoclaved in their Steritubes for 20 minutes under 20 pounds of pressure at 250° F., then allowed to vacuum dry for 10 minutes.

RESULTS

Healthy Individuals.—The stactometric ratio and hematocrit of each subject were recorded on an individual graph whose horizontal axis shows the date of each test while the vertical axis indicates the stactometric ratio and hematocrit. Figs. 2 and 3 are copies of 30, consecutive, daily tests on a 38-year-old man and a 24-year-old woman. Fig. 4 includes the distribution graphs of 1,800 stactometric ratio and 1,800 hematocrit test results on 237 healthy males and females. These were apparently normal individuals going about their daily routines; about 80 per cent of these subjects included interns, residents, nurses, medical students, and practicing physicians and surgeons. The ages varied from 18 to 90 years, with the average age at 30 years. Some were tested only once, while others were tested daily for 30 to 40 days, then every other day for 2 to 3 weeks, and at least 3 times a month for a year or more thereafter. The majority had at least 5, consecutive, daily tests. Some have been tested off and on for 6 years. Thus, samplings of normal individuals have been taken during the seasons of the year under the various conditions of everyday living, including the stresses of minor illnesses, frustrations, fatigue, tension, hard work, cold, heat, hunger, etc. The extremes of

*Sight Savers, Dow Corning Corp.

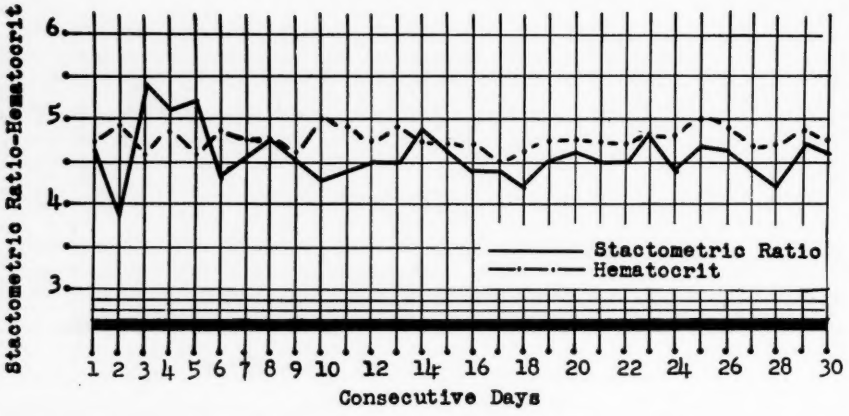


Fig. 2.—A copy of 30, consecutive, daily stactometric ratio and hematocrit test results on a 38-year-old normal man. The elevation of the stactometric ratio on days 3, 4, and 5 was during an upper respiratory infection. The standard deviation for the stactometric ratio is 0.31, for the hematocrit, 1.2, and for the stactometric ratio variation, 3.4.

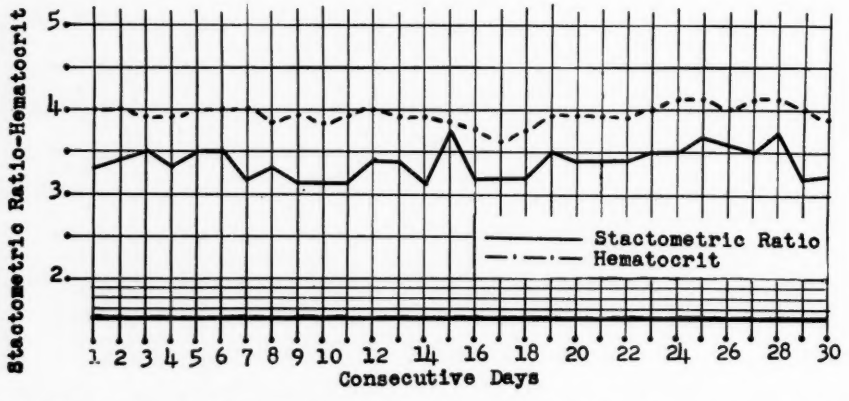


Fig. 3.—A copy of 30, consecutive, daily stactometric ratio and hematocrit test results on a 24-year-old normal woman. Note that the stactometric ratio tends to be lower in relation to the hematocrit than in the man. The slight peak on the fifteenth day was during mensis. The standard deviation for the stactometric ratio is 0.18, for the hematocrit, 1.2, and for the stactometric ratio variation, 1.6.

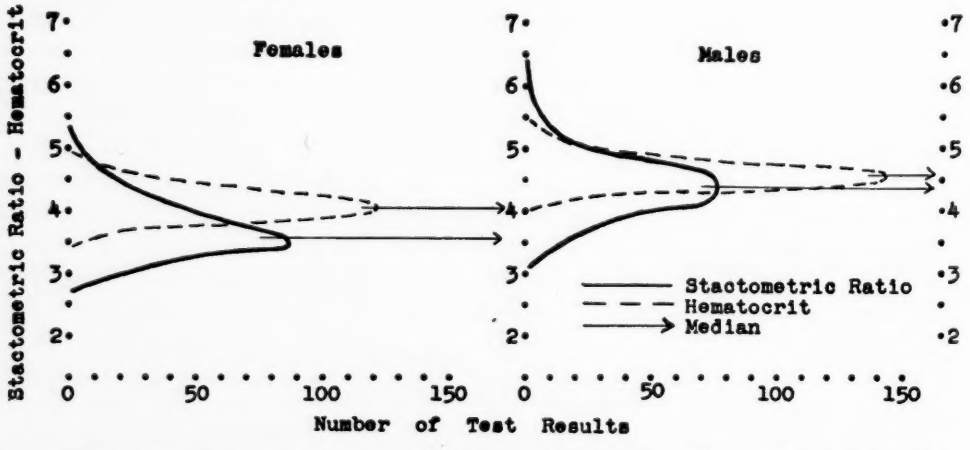


Fig. 4.—Distribution graphs of 1,800 stactometric ratio and 1,800 hematocrit test results on 237 normal, healthy individuals consisting of 890 tests on 112 females and 910 tests on 125 males. The general relationship of the stactometric ratio to the hematocrit for each group is indicated by the median arrows.

stactometric ratio fluctuations for the females were 2.8 to 5.4 with the median at 3.6, while the hematocrits ranged from 34 to 50 per cent with the median at 41 per cent. The stactometric ratios of the males varied from 3.1 to 6.5 with the median at 4.4, and the hematocrits ranged from 40 to 55 per cent with the median at 46 per cent.

As these data accumulated, it became apparent that the stactometric ratio under normal conditions of well-being was consistently below the hematocrit and rose above it when the individual was subjected to disturbing stimuli. This observation and the need to individualize and compare stactometric ratios in subjects having different hematocrits led to the formulation of the stactometric ratio variation. This consisted in making the hematocrit the base line, labeling it zero and expressing the stactometric ratio variations in terms of plus or minus the base line according to the following formula:

$$SRV = 10\ SR - H$$

in which SRV is the stactometric ratio variation, SR the stactometric ratio and H the hematocrit in per cent. Thus, 3 stactometric ratio values of 4.0, 4.5, and 5.0, each with a hematocrit of 45 per cent, would have stactometric ratio variations of –5, 0, and +5, respectively. (Occasionally, very viscid blood will be encountered, particularly in polycythemias, and the blood will have a very high stactometric ratio or may even clot before 14 drops are delivered.

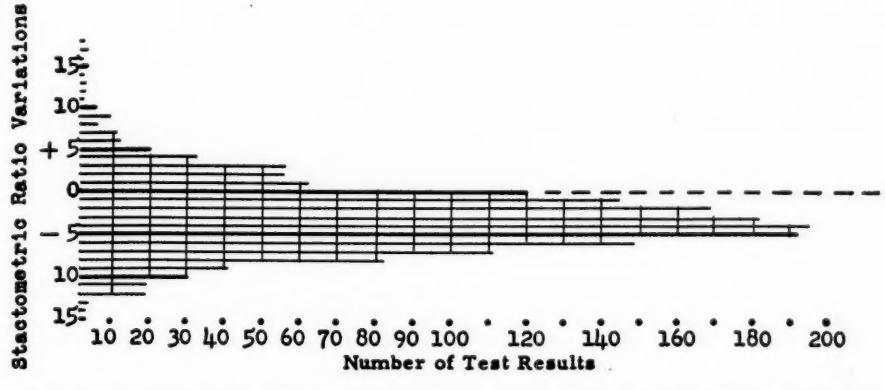


Fig. 5.—A distribution graph of the combined stactometric ratio results of Fig. 4 expressed in terms of stactometric ratio variations. Practically all the results above the 0-line were obtained when the subject was under the stress of a minor illness or sustained tension.

To facilitate charting, an arbitrary ceiling has been placed at a stactometric ratio of 10 or a stactometric ratio variation of +50.) Fig. 5 shows the combined results of Fig. 4 expressed in terms of stactometric ratio variations (hereafter abbreviated as SRV). The great bulk (86 per cent) of the SRV's are below the base line, and practically all of the results above the base line were those obtained when the subject was responding to some physical or mental alarm stimulus. The median SRV of the females is –5, that of the males –2; and that of the combined groups is –4. Females tend to approach the base line and occasionally go above it during a day of the menstrual period. SRV's in males tend to hug the base line more closely and go above it more frequently after the age of 40 years. The marked difference between

male and female SRV's disappears after the menopause when the female SRV's tend to become like those of males. Both males and females tend to show increases in their SRV's when the weather suddenly turns cold, e.g., a drop from a usual of 80° F. to 55° or 50° F. in several hours. Eighty per cent of the plus SRV's and all above +4 in Fig. 5 were contributed by males.

This group of healthy individuals seems to suggest that the normal equilibrium of the blood constitutents, during the state of well-being is represented by the minus SRV's. This was confirmed by the forthcoming patients who had plus SRV's when they were obviously sick and minus SRV's when they recovered.

A variation in results whose cause is not known is that occurring when an individual is subjected to 2 consecutive tests a few minutes apart. In about 90 per cent, the second test will show a lower stactometric ratio, a lower hematocrit by 1 to 3 per cent, and the blood will be brighter red in color. The drop in an abnormally high stactometric ratio on quick second-testing may be considerable and far out of proportion to the hematocrit decrease. Apparently, the first venipuncture causes changes that are reflected in the second test. A few patients having abnormally high, first-test stactometric ratios were subjected to quick second tests, and it was determined on the basis of clinical outcome that it is the result of the first test that is of prime importance. Practically all the SRV's presented in this study were obtained from one, daily, drop test venipuncture. The venipuncture itself seems to have considerable influence on the result in abnormal individuals; an imperfect vein entry may result in an extremely high stactometric ratio. Difficult venipunctures do not seem to influence the result markedly in normal individuals.

Patients.—The patients studied were those hospitalized for thromboembolism or for conditions likely to be complicated by thromboembolism. (The term thromboembolism is used in this discussion to refer to intravascular thrombosis with or without embolism; the occurrence of embolism will be mentioned in the specific instances.) They will be presented in an order that will help answer the following practical questions: (1) Will this patient, who has just undergone surgery or serious trauma, develop thromboembolism? (2) Is this patient with known thromboembolism likely to recover without further trouble, or will more thromboembolism develop? (3) Does this person presenting signs and symptoms suggestive of thromboembolism have something serious developing, or are his complaints of no serious consequence? The subjects have been arranged in the following groups: (1) Postoperative, posttraumatic, and post-partum. (2) Venous thromboembolism. (3) Cardioarterial thromboembolism. (4) Blood-cell abnormalities commonly complicated by thrombosis. (5) Miscellaneous.

Postoperative, Posttraumatic, and Post-partum.—This group included 128 patients involved in 143 stressing procedures. The majority were selected because they were, by clinical evaluation, likely candidates for thromboembolism because of age, extensive surgery or trauma, previous history of thromboembolism or presence of cardiovascular disease. The ages varied from 19 to

89 years, with an average age of 52 years. Thirty (23.4 per cent) had a past history of thromboembolism; 47 (36.7 per cent) had cardiac or vascular disease or both; 26 (20.3 per cent) had one or more of such secondary predisposing factors as varicose veins, vasospasm, diabetes, blood dyscrasia, thromboangiitis obliterans or cancer. The types of stressing events included: 18 gastrointestinal procedures, 22 lumbar sympathectomies, 12 thigh amputations, 11 hysterectomies, 12 fractured hips that were nailed, 2 fractured spines, 5 appendectomies, 10 exploratory laparotomies, 8 cholecystectomies, 7 herniorrhaphies, 5 post-partum patients, 3 splenectomies, 2 dorsolumbar sympathectomies, 4 prostatic resections, and 1 each of hemorrhoidectomy, transplantation of ureters, nephrectomy, excision of ruptured intervertebral disc, lobectomy, cesarean section, and a patient who was recovering after being struck by lightning.

These patients showed a frequently recurring sequence of SRV results that described a characteristic curve following major surgery or serious trauma. The pattern starts with a minus SRV on the first postoperative day and gradually increases daily until a plus SRV peak is reached on about the seventh day. Then it quickly returns to the base line by the tenth day. Fig. 6 is a scattergram of 79 patients on whom daily records were obtained from the first to the seventh postoperative days and a somewhat smaller number of patients whose records extended from the day of operation to the tenth postoperative day. The median is indicated by the dark line that follows the curve described. Fig. 7 includes copies of 2 case records from the day of operation to the tenth postoperative day. The day of operation results vary because of such immediate postoperative factors as shock, dehydration, blood loss, fluid replenishments, and other conditioning influences. This apparently normal postoperative curve is followed when the operation is the only recent, serious physical disturbance incurred by the patient. When surgery is superimposed during the course of an existing illness or follows a recent operation or injury, this first 10-day sequence of SRV's is not followed as such. It seems that the reaction caused by most recent alarming stimulus amplify, reduce, or somehow modify the reactive processes already present and result in a different daily sequence of SRV's. Even in such cases of multiple, staggered stresses, however, peaks of plus SRV's seem to be reached about every 7 days, with intervening lower plus and minus SRV's when the patient is serially tested for two weeks or more. Postoperative patients, who have been tested more than 10 days, have had these SRV undulations continue for 3 to 6 weeks. The physicochemical variations reflected by the foregoing SRV's conform to findings of more specific fluctuations of platelets,[4-6] coagulation time,[7] prothrombin time,[4, 5] heparin tolerance,[7] and fibrinogen.[5] They also parallel the blood sugar, lipids, cholesterol, albumin, fibrinogen, NaCl, and alkali reserve changes that occur during the Alarm Reaction and Stage of Resistance of Selye's General Adaptation Syndrome.[8] This normal postoperative curve has also been observed to follow myocardial infarction, pulmonary infarction, pneumonia, and venous or arterial thrombosis.

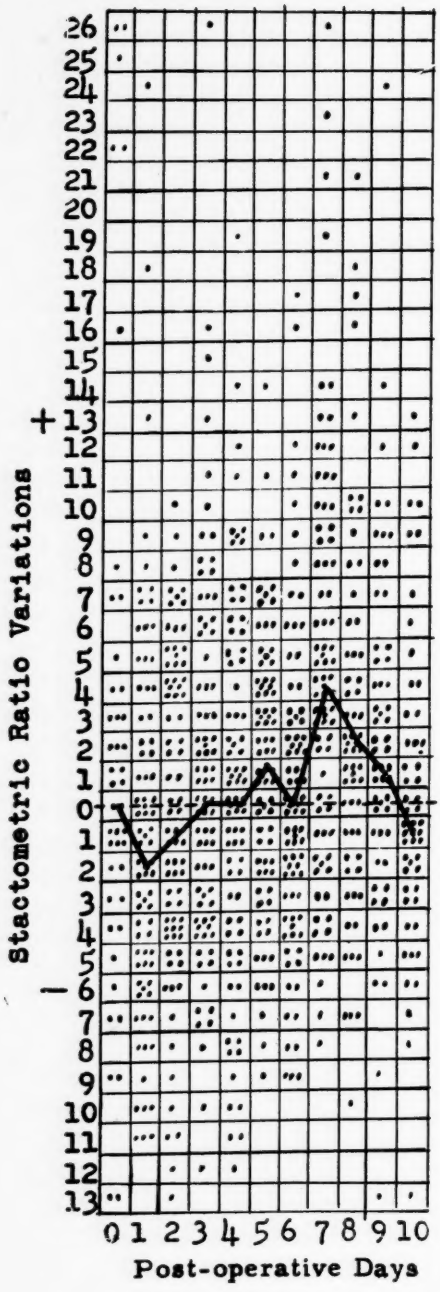


Fig. 6.

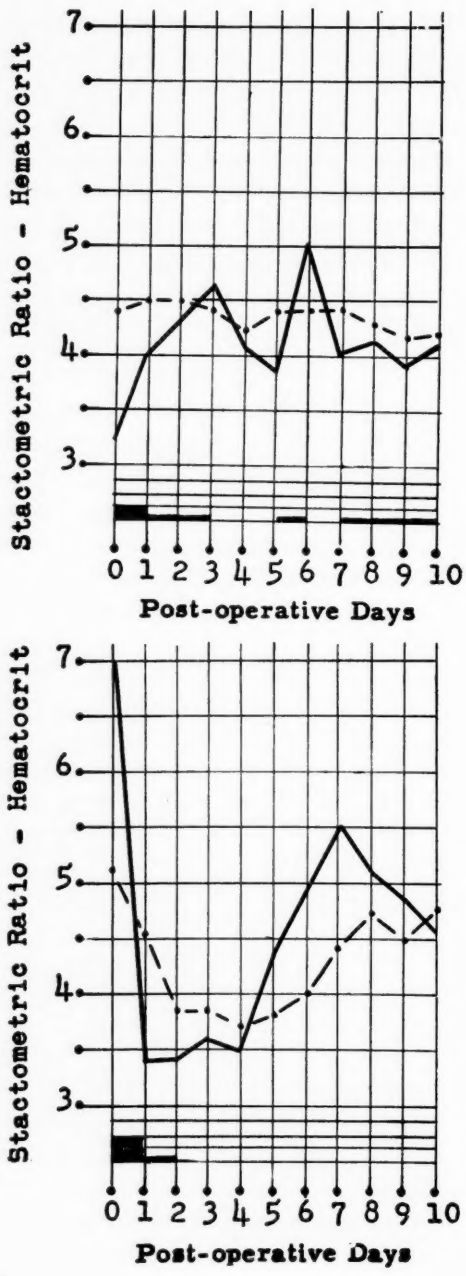

Stactometric Ratio - Hematocrit
7 6 5 4 3
0 1 2 3 4 5 6 7 8 9 10
Post-operative Days
Stactometric Ratio - Hematocrit
7 6 5 4 3
0 1 2 3 4 5 6 7 8 9 10
Post-operative Days

Fig. 7.

Fig. 6.—A scattergram of test results on 79 postoperative patients for 11 consecutive days. The dark line represents the median stactometric ratio variations and shows the progressive increase of plus variations until a peak is reached on the seventh postoperative day. This seventh-day peak was observed in 56 of the cases, which is statistically significant on the basis of the Sign Test.

Fig. 7.—Copies of stactometric ratio and hematocrit records on 2 postoperative patients showing the usual, normal variations after major surgery.

In this group of 128 patients, 12 (9.3 per cent) developed thromboembolism. Of these 12 cases, 5 developed only a superficial thrombophlebitis of no consequence. Six others were serious cases of deep or both superficial and deep venous thrombosis, 3 of whom had a fatal embolism and another a near-fatal embolism. One patient had a fatal, ascending arterial thrombosis of his femorals, iliacs, and aorta up to the renal branches.

The SRV's of the patients who developed serious thromboembolism showed major differences from the normal postoperative curve. All started off with a high plus SRV from the first postoperative day and they continued to have high plus SRV's until thrombosis occurred. The clinical appearance of thrombosis or embolism usually followed a sharp fall in the stactometric ratio. Fig. 8 is a copy of a record on this type of patient. It seems, then, that

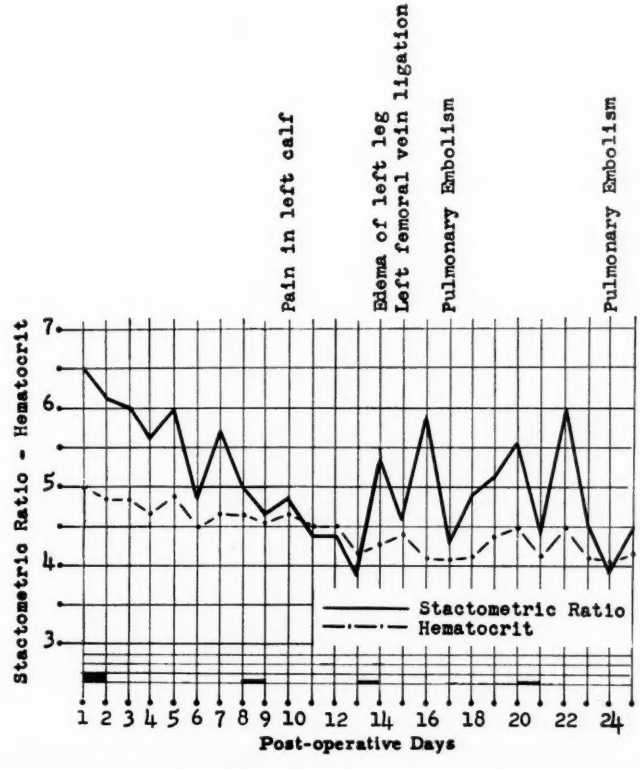


Fig. 8.—A copy of the stactometric ratio and hematocrit record on a 72-year-old man who had undergone a resection of the colon for carcinoma. This patient developed deep venous thrombosis and recurrent pulmonary embolism with a fatal outcome. Note the persistent elevation of the stactometric ratio after operation and the dips that preceded the clinical appearance of thrombosis or embolism. The postoperative average SRV is +11. The standard deviation for the stactometric ratio is 0.74, for the hematocrit, 2.6, and for the stactometric ratio variation, 5.9.

plus SRV's and their persistence is of significance. This and the observation that SRV's usually reached their peak in about 7 days suggested a working scheme for evaluating and comparing the cases studied. This consisted in adding the plus SRV's encountered in the first 7, consecutive, daily tests and dividing the sum by 7 to obtain the average plus SRV (e.g., SRV's for 7 con-

secutive days of –4, –2, +3, +4, –2, +5, and +5 gives a total of +17, which divided by 7 gives an average of +2). This average plus SRV was determined on all the patients in this and the other groups.

All the patients in this group who developed thromboembolism had an average SRV of +8 or more. The 5 patients who contracted superficial thrombophlebitis had average SRV's of +8, +8, +8, +8, and +9. The 7 cases of serious thromboembolism had average SRV's of +10, +11, +14, +14, +16, +16, and +18. Of the 128 patients, 26 (20.3 per cent) had an average SRV of +8 or more. Thus an average SRV of +8 or more carried a 46.1 per cent chance of incurring some form of thromboembolism in this group of patients. The serious cases of thromboembolism, that is, those who developed iliofemoral venous thrombosis, extensive arterial thrombosis, and fatal or near-fatal emboli, all had average SRV's of +10 or more. Only 14 (10.9 per cent) of the 128 patients had an average SRV of +10 or more, and these high average plus SRV's carried a 50 per cent chance of incurring serious thromboembolism with a 28.5 per cent mortality. Three patients who had average SRV's of +10 or more without thromboembolic complications had external biliary fistulas whose closures were followed by the prompt return of the SRV's to normal. It was also noted that the presence of gangrene or diabetes tended to be associated with SRV's that were close to or above the dangerous range. Two patients who died from massive pulmonary embolism gave no clinical warning of what was impending, and their SRV's were +10 or more for 5 days before the fatal embolism. The other thromboembolic deaths were 24 and 26 days after operation.

Venous Thromboembolism.—This group of 226 includes patients having venous thrombosis with or without embolism and patients suspected of having venous thrombosis or pulmonary embolism. They are subdivided as follows:

1. Deep venous thrombosis: These were 102 patients who obviously had occlusion of the iliofemoral veins as indicated by pain, tenderness and swelling of the involved extremity. Twenty-nine (28.4 per cent) had already sustained pulmonary emboli when the studies were started. Fifteen (14.7 per cent) developed more thrombosis while under observation, and 11 (10.7 per cent) had pulmonary emboli which were fatal in 3 (2.9 per cent of this subgroup, 27.2 per cent of the emboli). Of the 29 who already had pulmonary emboli when the studies were started, 10 (34.4 per cent) had more thromboembolism which was fatal in 3 (30.0 per cent).

2. Superficial venous thrombosis: These were 36 patients whose superficial veins were involved in an obvious inflammatory process with the vein being indurated, tender, and surrounded by an area of erythema without swelling of the involved extremity. None had any pulmonary emboli. Three (8.3 per cent) had more thrombosis in the same vein or in another superficial vein.

3. Superficial and deep venous thrombosis: Thirteen patients had superficial venous thrombosis which extended to include the deeper veins (tibials, popliteal, femoral, and iliac) resulting in swelling of the involved extremity.

Two (15.3 per cent) had already sustained pulmonary emboli when first seen. Five (38.4 per cent) had more thrombosis, and 2 had pulmonary embolism during the course of observation.

4. Pulmonary infarction source unknown: These were 11 patients with clinical and x-ray evidence of pulmonary infarction from emboli whose source was not found. Five (45.4 per cent) sustained more embolizations which caused the death of one patient.

5. Signs and symptoms suggestive of venous thrombosis: These were 53 hospital patients seen in consultation because of pain and tenderness in the calf of the leg strongly suggestive of venous thrombosis. Thirteen (22.4 per cent) had a positive Homan's sign. Two (3.7 per cent) subsequently showed more definite signs of venous thrombosis, and one patient had a sudden, fatal pulmonary embolism 3 weeks after a hysterectomy.

6. Signs and symptoms suggestive of pulmonary infarction: These were 11 patients who had sudden, pleuritic chest pain without other clinical or x-ray evidence of pulmonary infarction. None showed any further evidence of pulmonary embolism or peripheral thrombosis.

Thirty-three patients (14.6 per cent of the group, 20.3 per cent of the cases with proved thromboembolism) developed more thrombosis or embolism while under observation. Thirty (90.9 per cent) of these had average SRV's of +7 or more (the 3 exceptions showed no plus SRV's at all, and 2 of them were very ill and died). Sixty-three (27.8 per cent of the group, 38.6 per cent of the cases with proved thromboembolism) had average SRV's of +7 or more. Thus, an average SRV of +7 or more carried a 47.6 per cent chance of incurring more thromboembolism, while an average SRV below +7 meant a 98.2 per cent chance of making an uneventful recovery in this group of patients. Another significant point was demonstrated by 11 patients with average SRV's from +10 to +36, who had recurrent thrombosis or embolism for 3 to 5 months after the initial attack. During this time their SRV's remained abnormally high, while, clinically, they seemed well enough to resume work and have have therapy discontinued. It was evident in these patients, as well as in later groups, that it was not safe to discontinue therapy as long as the SRV's remained abnormally high.

Cardio-arterial Thromboembolism.—These were 172 patients whose heart or arterial disease was due to or likely to be complicated by thromboembolism. Included in this division were:

1. Myocardial infarction: Forty-four patients had electrocardiograms which indicated acute myocardial infarction when the studies were started. Six (13.6 per cent) of these patients developed clinical thromboembolism. There were 6 (13.6 per cent) fatalities (2 were due to cerebral emboli, one to a ruptured heart whose ventricles contained multiple thrombi, one to refractory congestive failure, and the remaining 2 due to undetermined causes). All those who showed clinical thromboembolism and all the fatalities, except the case of congestive failure, had an average SRV of +7 or more.

2. Substernal pain suggestive of myocardial infarction: Sixty patients, whose ages averaged 51 years, were studied because of substernal pain strongly suggestive of coronary insufficiency or myocardial infarction and showed no abnormalities in the initial electrocardiogram. Subsequent electrocardiograms taken at about weekly intervals showed definite myocardial infarction from one to 4 weeks later in 12 (20 per cent) of them. All the patients who showed subsequent evidence of myocardial infarction had an average SRV of +7 or more. None of those having an average SRV below +7 showed any evidence of myocardial damage after a follow-up of at least 2 months.

3. Long-term follow-up on recovered myocardial infarction: These were 5 patients who had recovered from myocardial infarction and had returned to their usual work; 3 of these patients also had histories of recurrent thromboembolism. They were tested about once a week to determine if there might be SRV warning signs of impending myocardial infarction or thromboembolism. These patients have been under observation for 2 to 5 years. Two sustained new myocardial infarctions while under observation, and their SRV's for the 3 tests before the attack (at least 3 weeks) averaged +10 and +14. One died soon after the attack. Before his fatal coronary occlusion, this 53-year-old diabetic sustained, during the 3 years he was observed, a right hemiplegia with almost complete recovery, a left hemiplegia with only partial recovery, another right hemiplegia with recovery, tibial vein thrombosis and several minor illnesses and mental stresses. Preceding and accompanying all these episodes were exaggerated SRV's. In fact, this patient had intervals of only 1 or 2 months of minus SRV's. The other 3 patients, who have remained relatively well, had no average of over +7 on 3 consecutive weekly SRV readings. The surviving patients had periods of several months when the SRV's were usually normal with occasional increases when under stress. All showed marked increases when the weather suddenly turned cold. Fig. 9 is a long-range record in terms of SRV's on a patient who is generally representative of this group. It has been noted that these patients tend to show exaggerated SRV's in response to stress as compared to normal individuals. But, here again, trouble resulted when the SRV's were persistently high. No adverse effects were noted when the SRV's were normal or when there was an occasional, nonpersistent, abnormally high SRV. The SRV determination has served as a helpful guide in warning these patients of impending danger during periods of stress and getting them to slow down and eliminate or control the responsible stressors.

4. Auricular fibrillation: Twenty-one patients, subdivided into those who already had an embolus when the studies were started (15 patients) and those who had no history of emboli (6 patients), had this arrhythmia. Nine (60 per cent) of the 15 patients having emboli had recurrent embolization which was fatal in 7 (77.7 per cent). All the patients having recurrent emboli, fatal or nonfatal, had an average SRV of +8 or more. The 6 who made uneventful recoveries despite a history of embolization had an average SRV of +6 or less.

None of the 6 patients who gave no history of embolism at any time developed thromboembolism, and their average SRV's were +3 or less. The combination of auricular fibrillation, embolization, and abnormally high SRV's was found to carry the highest mortality of the groups observed in this study.

5. Cerebral vascular accident: There were 17 patients who showed evidences of cerebral vascular disturbances ranging from transient irritation to hemiplegia. On the basis of history, mode of onset, presence or absence of hypertension, spinal tap findings, x-ray examinations, and clinical course, these patients were tentatively subdivided into the following types: hemorrhage, 5 (average SRV 0, 0, 0, +2, +4); thrombosis, 5 (average SRV +4, +8, +9, +10, +25); vascular spasm, 6 (average SRV 0, 0, 0, +2, +3, +8); metastatic carcinoma, 1 (average SRV 0). All that can be said of these few cases is that those suspected of having cerebral thrombosis seem to have higher average SRV's, and those having higher average SRV's seem to have a poorer prognosis.

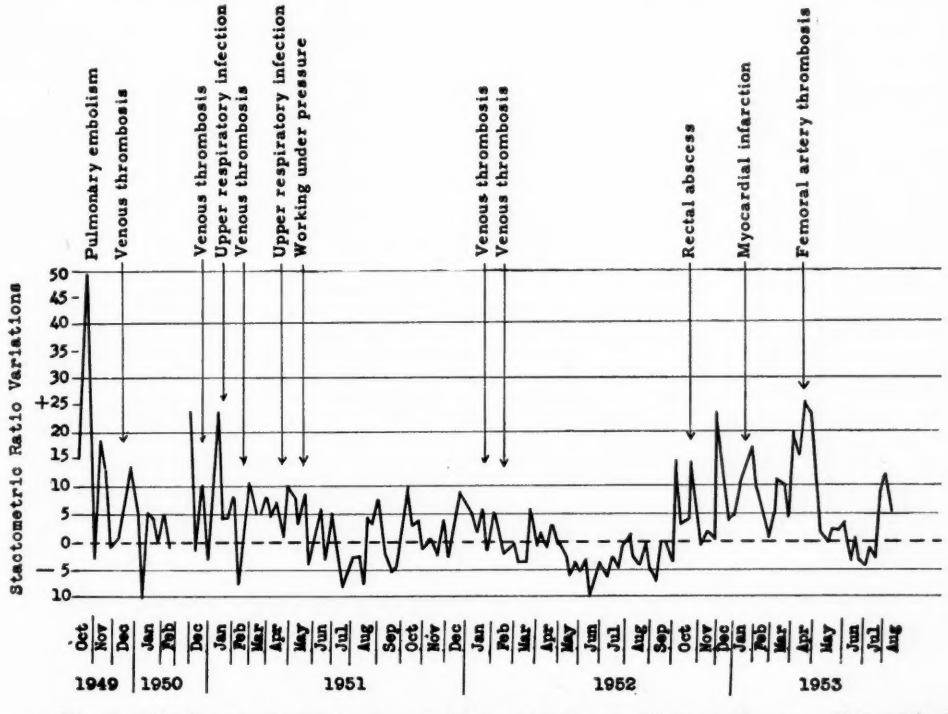


Fig. 9.—Weekly stactometric ratio variation record on a 63-year-old man. This patient had a past history of myocardial infarction and recurrent thromboembolism for 10 months, 12 years before this record was started. Note the increases in stactometric ratio variations in relation to the stresses of illness, mental strain, and the cold months.

6. Congestive heart failure: These were 12 patients whose congestive failure was not a complication of the preceding groups mentioned. The significant observation from these patients is the marked effect of intensive diuresis on the viscosity of the blood. Five of these patients had hematocrit increases of 8 to 10 per cent following the enthusiastic use of diuretics which resulted in an acute rise in the SRV's to the +20's. Four of these dehydrated

patients developed venous or arterial thrombosis, and 2 of them had near-fatal pulmonary emboli. These cases of forced diuresis resulting in marked increases in blood viscosity are part of the evidence that sudden increases in the concentration of the cellular components of the blood will cause serious alterations in the physical state of the blood and result in tissue anoxia or thrombosis. Here again, all those who developed thromboembolism had high average SRV's (+12 or more).

7. Bacterial endocarditis: Two patients with this disease had multiple, small peripheral emboli. Both made good recoveries. Their average SRV's were 0 and +3.

8. Thrombo-angiitis obliterans: These were 5 male patients, 2 of whom were observed through the acute, subacute, and quiescent phases of the disease. The average SRV's were high (+13 and +9) when thrombosis was occurring during the acute and subacute phases, and fell back to a high-normal range during the quiescent stage.

9. Arterial aneurysm: These were 2 males, one with an abdominal aortic aneurysm and the other with a thrombosed popliteal aneurysm which required amputation. Their average SRV's were 0 and +1.

10. Peripheral arterial thrombosis: Four patients not included in any of the foregoing groups had thrombosis of the femoral or iliac arteries due to atherosclerosis. Three recovered and one, with extensive thromboses on large atherosclerotic ulcers in his femoral and iliac arteries and lower aorta, died. Their average SRV's were 0, 0, +2, +4.

Forty-one patients (23.8 per cent) of this cardioarterial group developed thromboembolic complications or myocardial infarction resulting in death for 17 (41.4 per cent) while under observation. Thirty-seven (90.2 per cent) of these patients had average SRV's of +7 or more (the 4 exceptions being 2 cases of bacterial endocarditis, one case of extensive arterial thrombosis, and one case of myocardial infarction with refractory congestive failure). Fifty-four (31.3 per cent) of the entire group of 172 patients had average SRV's of +7 or more. Thus, an average SRV of +7 or more carried a 64.8 per cent chance of incurring serious thromboembolic complications or myocardial infarction with an over-all mortality of 39 per cent, while an average SRV below +7 meant a 96.7 per cent chance of avoiding such complications.

Blood-Cell Abnormalities Likely to Be Complicated by Thrombosis.—There were 26 patients having blood-cell abnormalities reputed to carry a high incidence of thrombosis. They include:

1. Primary polycythemia, 5 patients.

2. Secondary polycythemia: Polycythemia in these 13 patients was apparently due to pulmonary disease (3 cases), congenital heart disease (1 case), excessive blood transfusions (3 cases), and stress (6 cases). The polycythemias of stress[9] are individuals who, in response to stress, have 10 to 15 per cent increases in their already high-normal hematocrit levels and develop symptoms of acute polycythemia.

3. Thrombocytosis: This group was composed of 5 patients whose platelet counts varied from 700,000 to 1,400,000 during the period of observation.

4. Sickle-cell anemia: Three patients had this disease.

Of the 26 patients in this group, 22 (84.6 per cent) had average SRV's of +7 or more, and 15 (57.6 per cent) of these had a history of thrombosis or developed thrombosis which was fatal in 4 (26.6 per cent) while under observation.

Hematocrits of 55 per cent or more were not associated with minus SRV's despite the subjects apparent stress-free state. The degree of plus SRV's with high hematocrits depends not only on the hematocrit elevation but also on its duration. Acute increases in the hematocrit to 55 per cent from high normal values of 48 to 50 per cent will result in rise of SRV's to highest levels (+40 to +50). The highest average SRV's (+43 and +47) obtained in this study were those due to excessive blood transfusions that raised the hematocrits to 59 and 63 per cent. On the other hand, a long-standing hematocrit of 55 to 60 per cent may have SRV's from +5 to +10 under stress-free conditions.

The impression gained from observation of patients with blood-cell abnormalities is the marked disturbance in the physical properties of the blood when there is an absolute or relative increase or a qualitative variation in the red blood corpuscles. This disturbance results in an actual burden on circulation, causes clinical evidence of tissue anoxia, and not only was partially responsible for the fatalities in the above patients, but also played a highly suspicious role in 5 other cases not recorded in this group because only one or 2 tests were possible before death. These results help fortify the belief that narrowed, sclerotic arteries and very viscid blood do not provide for adequate tissue nutrition.

Miscellaneous.—This group of 27 patients had conditions not uncommonly complicated by thromboembolism or had recently recovered from some form of thromboembolism. Included were 8 patients who were very ill, 5 of whom had intra-abdominal abscesses, 2 acute pancreatitis, and 1 a severe pneumonia. All 8 had average SRV's of +8 or more, and all made good recoveries without thromboembolic complications. The other 19 patients included 4, 5, and 8 weeks' recoverd myocardial infarctions, recovered cerebral vascular accidents, recovered venous thromboembolism, a bedridden, recent paraplegic, diabetics with foot infections and peripheral arterial insufficiency due to arteriosclerosis. None of these had abnormal elevations in their average SRV's.

These miscellaneous cases serve to emphasize the impression that SRV's reflect response to stress which varies not only with the individual but also with the stage of the disease and the presence of conditioning factors such as large pus pockets, abnormal blood cells, or abnormal metabolites.

One or 2 tests were obtained on 184 patients who, for various reasons, could not be serially tested. They include the same types recorded in the foregoing groups. They were simply subdivided into those who had an SRV

of less than +7, those with an SRV of +7 or more, and those of either group who died. Of the 184 patients, 153 (83.1 per cent) had SRV's less than +7 and only 7 (4.5 per cent) of them died, while 31 patients (16.9 per cent) had SRV's of +7 or more and 8 (25.8 per cent) of them died.

These single-test patients seem to indicate that the chance of a serious case showing an abnormally high SRV on the first test is quite good. As noted time and again, however, in patients serially tested for about 7 days, the SRV will drop to normal levels after a thrombosis or embolism only to increase to abnormal levels on subsequent days; and an abnormal SRV obtained about 7 days after a known serious stress may drop and remain at normal levels thereafter. Thus, it is not recommended that patients be evaluated on the basis of one test.

DISCUSSION

Of the 1,000 individuals sampled, 237 were healthy, normal subjects who were studied to determine the normal range of SRV's. They indicated that the SRV's are on the minus side of the base line (the hematocrit) during the state of well-being and on the plus side during responses to alarming stimuli. The remainder of the 1,000 was made up of 763 patients who were actively or recently involved in conditions commonly complicated by thromboembolism. These patients provided the important fact that serial, daily SRV determinations were necessary for the reliable evaluation of a case, and brought into use the 7-day average plus SRV. Five hundred and seventy-nine patients were available for average SRV determinations; they are summarized in Table I. It was found that 100 patients (17.4 per cent) developed thrombo-

TABLE I. SUMMARY OF 579 PATIENTS HAVING THROMBOEMBOLISM OR CONDITIONS LIKELY TO BE COMPLICATED BY THROMBOEMBOLISM ON WHOM AVERAGE PLUS SRV'S WERE DETERMINED

TYPE OF CASE	CASES (NO.)	AVERAGE SRV		UNEVENTFUL RECOVERY		COMPLICATIONS					
						THROMBO-EMBOLISM		MYOCARDIAL INFARCTION		FATAL*	
		+6 OR LESS	+7 OR MORE	+6 OR LESS	+7 OR MORE	+6 OR LESS	+7 OR MORE	+6 OR LESS	+7 OR MORE	+6 OR LESS	+7 OR MORE
Postoperative post-traumatic	128	98	30	98	18	None	12	None	None	None	4
Venous thrombo-embolism	226	163	63	161	33	2	30	None	None	2	5
Cardioarterial thromboembolism	172	118	54	115	16	3	24	None	14	2	15
Blood-cell abnormalities	26	3	23	3	8	None	15	None	None	None	4
Miscellaneous	27	19	8	19	8	None	None	None	None	None	None
Total	579	401	178	396	83	5	81	None	14	4	28

*The fatalities are those directly or indirectly due to thromboembolism or myocardial infarction.

embolism or myocardial infarction that was fatal in 28 per cent while under observation. Of these 100 patients, 95 (95 per cent) had average SRV's of +7 or more. On the other hand, only 5 (1.2 per cent) of the patients with average SRV's below +7 developed such complications, and these 5 patients did not have positive average values. It seems, therefore, that an average SRV of +7

is the dividing line in values between those prone to develop thromboembolism or myocardial infarction and those likely to make good recoveries. Thus, an average SRV of +7 or more carried a 53.4 per cent chance of incurring thromboembolism or myocardial infarction with a mortality of 29.4 per cent, while an average SRV of less than +7 meant a 98.1 per cent chance of avoiding such complications. The obvious significance of these results is further confirmed statistically by a calculation of the Youden J index* for rating diagnostic tests.[10]

Since 439 (75.8 per cent) of the 579 patients had plus SRV's while manifesting clinical signs and symptoms of disturbed physiology, it might be presumed that there is some relation between plus SRV's and the body's reaction to stressing stimuli. This presumption and the finding that the complications studied fell in 2 of 3 readily formed groups suggested the further subdivision according to stress reaction for better prognostic and diagnostic guidance. Postulating that the plus SRV is the normal response to stress and the complications represent evidence of either intense or deficiency of reaction, the patients can be subdivided into stress normoreactor, stress hyperreactor, and stress hyporeactor groups (Table II). Since 95 per cent of the

TABLE II. PROGNOSIS DISTRIBUTION OF 579 PATIENTS OF TABLE I ACCORDING TO STRESS REACTION TYPE

GROUP	CASES (NO.)	PER CENT OF TOTAL	PER CENT OF GROUP		
			UNEVENTFUL RECOVERY	COMPLICATIONS	
					FATAL
Normoreactor (ASRV +1 to +6	261	45.1	100.0	None	None
Hyporeactor (ASRV 0)	140	24.2	96.5	3.5	66.6
Hyperreactor (ASRV +7 or more)	178	30.7	46.6	53.4	29.4

The average plus stactometric ratio variation is abbreviated as ASRV.

complications occurred at average SRV +7 or more, lines of division for the stress normoreactor and the stress hyperreactor occur at average SRV +1 and average SRV +7, respectively. Thus, the stress normoreactor group would include those from average SRV +1 to average SRV +6, while the stress hyperreactor group would include those with average SRV +7 or more. It will be noted (Table II) that the stress normoreactor group of 261 patients had no complications while the stress hyperreactor group of 178 patients had a 53.4 per cent incidence of complications.

The stress hyporeactor group is made up of 140 (24.2 per cent) patients who did not have average SRV's on the plus side because of low and infrequent plus SRV's or complete absence of plus SRV's. All these patients were labeled as average SRV 0 for this report because that is how their 7-day average plus SRV came out. Averaging their *minus* SRV's for 7 consecutive days gives results from SRV 0 to SRV −9. This group requires further study and may prove valuable from a diagnostic point of view. These patients presented certain clinical characteristics in common. They tended to have superficial

*J=0.777 with a 95 per cent confidence interval from 0.732 to 0.822 (J varies from 0 for a worthless test to 1 for a perfect test).

thrombophlebitis, arthritis, bursitis, tenosynovitis, retinitis, generalized "painful muscles," easy fatigability, easy bruiseability, almost continuous malaise, and strong allergic history. Many of these complaints were present simultaneously in the same patient at the same time, or were singly manifest in the same patient at different times. The suggestion seems to be that the stress hyporeactors are those prone to develop connective tissue disease. Some, who subsequently received cortisone therapy, showed increases in the SRV's to normal stress reactor range. It was also noted that a very ill patient with persistently minus SRV's had a poor prognosis; this combination was present in 3 patients with thromboembolic complications who did not survive. This group included 5 patients who developed recrudescence of thromboembolism, 3 of which were fatal, and a patient with myocardial infarction who died of congestive failure, which gives a mortality of 66.6 per cent in these 6 cases. As a whole, however, 96.5 per cent of the hyporeactors made uneventful recoveries.

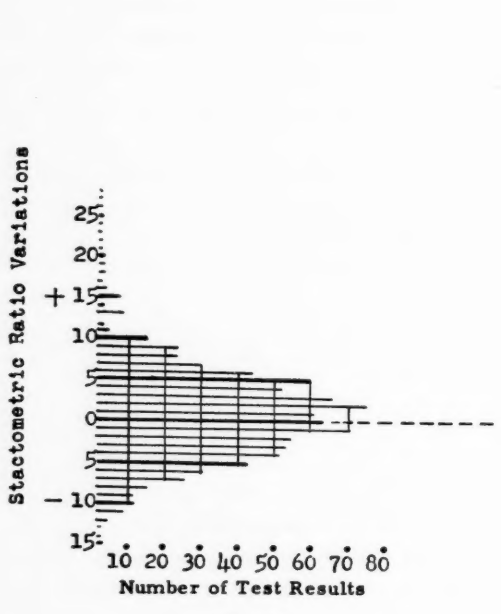


Fig. 10.

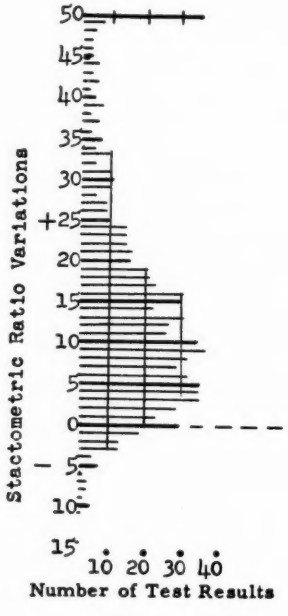


Fig. 11.

Fig. 10.—A distribution graph of 890 stactometric ratio variations on 97 patients with an average age of 54 years who had undergone serious physical stress (major surgery, trauma, myocardial infarction, venous thromboembolism) and made uneventful recoveries. The median stactometric ratio variation is +1.

Fig. 11.—A distribution graph of 877 stactometric ratio variations on 85 patients with an average age of 54 years who had undergone the same stresses as the patients in Fig. 10 and developed thromboembolism while under observation. The median stactometric ratio variation is +9.

Fig. 5, 10, and 11 graphically illustrate the striking increase in the SRV tide in response to stress. Fig. 10 represents the stress normoreactor group, while Fig. 11 shows the high excursions of the stress hyperreactors. The relation of the general effect of physical or emotional stress on the physicochemical state of the blood and its relation to thrombophlebitis has also been noted by Schneider and Zangari.[11, 12]

SUMMARY

The foregoing findings indicate that the physicochemical state of the blood as reflected by the drop test may fluctuate considerably in the same individual, and the range of fluctuation may vary markedly in different individuals. These variations seem to be related to stress reactions, with the suggestion that they are in direct proportion to the intensity of the reaction further modified by abnormalities in total blood-cell fluctuations, plasma protein and metabolite changes, and unknown factors resulting in an instability of the normal colloid state of the blood. The variations may be grouped in relation to stress response as stress-free, normal stress reaction, stress hyperreaction and stress hyporeaction. The stress-free SRV's are on the minus side and occur during the state of well-being; the normal stress reaction SRV's are from +1 to +6; the stress hyperreaction SRV's are +7 or more;* the stress hyporeaction SRV's average on the minus side despite the known occurrence of stress. In the 579 patients studied, uneventful recovery occurred in all of the stress normoreactor group, in 96.5 per cent of the stress hyporeactor group, and in 46.6 per cent of the stress hyperreactor group. The stress hyperreactor group included 95 per cent of the patients who developed thromboembolism or myocardial infarction and carried a 53.4 per cent chance of incurring such complications.

This presumptive evidence suggests a practical approach to the problem of anticipation and treatment of thromboembolism and myocardial infarction based on SRV's considered in relation to estimated vascular damage and blood flow. Briefly, SRV's would serve as a guide for the management of a patient undergoing a serious stress likely to be complicated by thromboembolism or a patient whose complaints are due to or suggestive of cardiovascular disease. Aggressive treatment would be indicated in those having average SRV's of +7 or more, while a more conservative course would be followed in those with average SRV's below +7.

CONCLUSIONS

1. A drop test is described that includes the combined effects of the viscosity, surface tension and specific gravity of the blood in one result called the stactometric ratio.

2. The impressions from more than 10,000 stactometric ratio variations in 1,000 individuals are discussed with particular reference to their prognostic value in thromboembolism.

3. The results suggest that stactometric ratio variations are reflections of the physicochemical variations of the blood in response to stress and can be divided into stress-free, stress normoreactor, stress hyperreactor, and stress hyporeactor groups.

4. Uneventful recovery occurred in all the patients in the stress normoreactor group and in 96.5 per cent of the stress hyporeactor group.

*This may have to be modified for hematocrits of 35 per cent or less.

5. The stress hyperreactor group included 95 per cent of the patients who developed thromboembolism or myocardial infarction while under observation and carried a 53.4 per cent chance of incurring such complications with a 29.4 per cent mortality.

I am grateful to Mr. Joseph M. Cameron of the Statistical Engineering Laboratory, National Bureau of Standards, Washington, D. C., for his statistical evaluation of the data on which the conclusions of this report are based.

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SURVIVAL OF RADIOCHROMATE-LABELED PLATELETS IN RABBITS

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PLATELET survival of 4 to 6 days has been reported in thrombocytopenic subjects transfused with whole blood, platelet-rich plasma, and platelet concentrates.[1-4] This method of measuring the life span of platelets cannot be used in subjects with normal platelet counts. Platelets labeled in vivo by feeding animals C^{14}-labeled formate have a similar survival time.[5] This method of labeling platelets is not applicable to man because of radiation hazard. In vitro labeling of platelets has been successful using P^{32} (ref. 6), I^{131} (ref. 7), and Cr^{51} as chromic chloride,[8] but the platelets thus labeled do not survive on reinjection, due to the trauma of the labeling procedures.†

This paper describes a method for labeling rabbit platelets with Cr^{51} as sodium chromate, and the survival of the labeled platelets in normal rabbits and rabbits made thrombocytopenic by injection of human serum.[9]

MATERIAL AND METHODS

1. *Obtaining Platelets.*—One hundred milliliters of pooled rabbit heart blood was collected with 0.5 ml. of 10 per cent sequestrene solution per 10 ml. of blood as the anticoagulant. One milliliter of a 2 per cent Triton WR1339 solution and 1 ml. of a 6 per cent high molecular-weight dextran solution were added per 10 ml. of blood. Separation of platelet-rich plasma was achieved by allowing the blood to stand 30 minutes at room temperature, centrifuging it at 1,000 r.p.m. for 5 minutes and pipetting off the plasma. A chilled‡ clinical centrifuge was used throughout. Final concentration of platelets was obtained by centrifuging the platelet-rich plasma once at 2,500 r.p.m. for 30 minutes, removing the supernatant plasma and resuspending the platelets in 2 to 5 ml. of pooled plasma.

2. *Cr^{51} Tagging.*—One hundred to 350 microcuries (0.1 to 0.5 ml.) of sodium chromate^{51} were added to the platelet suspension. The mixture was allowed to stand at room temperature 1½ hours; the platelets were then uniformly resuspended in 5 to 10 ml. of plasma and centrifuged at 2,500 r.p.m. for 30 minutes. After removal of supernatant plasma containing unbound radioactivity, platelets were resuspended in plasma.

3. *In Vitro Studies.*—Clot retraction studies were done as previously described[7] on tagged platelets washed in 10 ml. of plasma. Platelet counts were performed using 3.8 per cent sodium citrate solution in a flat-bottomed hemocytometer chamber with a phase microscope.

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†Since this paper was submitted, survival of platelets labed in vitro with P^{32} after reinjection in man has been reported. (Desai, R. G., Small, W., and Mednicoff, I.: J. Clin. Invest. 34: 390, 1955.)

‡Metal cups containing ice were placed in alternate positions with cups containing samples.

To determine the amount of transfer of radioactivity in vitro, chromate-labeled platelets were stored at 5° C. in a suspension of red cells in plasma for 18, 44, and 68 hours. In six experiments for each period, 1.5 ml. of plasma suspensions of washed labeled platelets containing 1-3 × 10^9 platelets (platelet radioactivity of 2,000 to 3,000 counts per second) were added to 5 ml. packed red cells and 1.5 ml. platelet-free plasma. Platelet counts were done before and after storage. Following storage, the mixture was centrifuged twice at 1,000 r.p.m. for 5 minutes to remove most of the platelets with the supernatant plasma. The remaining red cells were washed twice with 7 ml. of physiologic saline solution. The platelets were separated from the supernatant plasma and saline washes by centrifugation at 2,500 r.p.m. for 30 minutes. Determinations of platelet numbers and radioactivity were performed on all fractions.

4. *In Vivo Studies.*—Suspensions of tagged platelets washed only once with plasma were injected into adult rabbits through an ear vein. The elapsed time between withdrawal of blood for platelet labeling and injection of tagged platelets was 4 to 5 hours. Cardiac blood samples were withdrawn at intervals after injection of labeled platelets. Platelets were separated from red cells and plasma by centrifugation at 1,000 r.p.m. for 5 minutes, followed by plasma centrifugation at 2,500 r.p.m. for 30 minutes and resuspended in 1 ml. of water. Aliquot samples of platelets, plasma, and red cells were assayed for radioactivity. A well-type scintillation crystal was used to assay wet samples in Lusteroid tubes.

In 9 normal animals the survival of labeled platelets was studied by obtaining blood specimens at 2, 20, 44, 68, 92, and 120 hours after the injection of labeled platelets.

In 10 animals, 22 hours after injection of labeled platelets, 10 to 15 ml. of serum from healthy human subjects were injected intravenously to produce thrombocytopenia.[9] This experiment was repeated in 5 splenectomized animals. Five normal rabbits injected with rabbit serum served as controls. Animals injected with human serum died with massive pericardial hemorrhage following the first or second cardiac puncture after serum injection. Control rabbits injected with rabbit serum were killed by a blow on the head at a time interval corresponding to the death of the experimental animals. Autopsies were performed immediately; spleens, hearts, kidneys, and samples of liver, lung, and muscle were homogenized in 25 per cent sodium hydroxide and assayed for radioactivity. Bone samples were treated with 89 per cent formic acid prior to assay.

Five rabbits were injected intravenously with sodium chromate^{51} in platelet-free plasma comparable in radioactivity to that injected with platelets and plasma in the preceding experiments. Blood samples were obtained in 3 animals of this group after 20 hours and assayed for radioactivity in the platelet, plasma, and red cell fractions; in 3 animals assay of the organs was performed 24 hours after injection. In 5 rabbits sodium chromate^{51}-tagged red cells were injected intravenously and organ assay was performed 24 hours after injection.

RESULTS

1. *In Vitro Studies.*—Table I shows the mean final platelet radioactivity and radioactivity of 3 plasma washings in total counts per second (c.p.s.) for 29 radiochromate tagging procedures. In every experiment the radioactivity remaining in the platelet fraction was more than 10 times the radioactivity of the final plasma washing. The percentage of total added radioactivity which remained in the platelet fraction varied from 1.0 to 18.6 and averaged 6.9. (In the earlier experiments the values obtained were low and improved as familiarity

TABLE I. MEAN RADIOACTIVITY (TOTAL COUNTS PER SECOND) OF SUCCESSIVE PLASMA WASHINGS OF CHROMATE-TAGGED PLATELETS AND FINAL PLATELET RADIOACTIVITY IN 29 TAGGING PROCEDURES

First wash	246,790
Second wash	16,231
Third wash	954
Platelets	20,000

with the technique increased.) The tagged platelets washed once with plasma promoted clot retraction well in platelet-poor plasma, a function which decreased with repeated washings.

Table II shows the recovery of platelets and radioactivity in various fractions following the storage of tagged platelets with red cells in plasma. There was a gradual loss of radioactivity from the platelets and increase of red cell radioactivity during the storage period. At 68 hours a total of 66.5 per cent of the initial radioactivity was still present in the platelets recovered from plasma and washings; the red cell fraction contained 17.6 per cent of the total radioactivity. The remaining radioactivity was present in the plasma and saline washes. Since approximately 95 per cent of the original platelets were still present by count at 68 hours, the radioactivity in the red cells and plasma fractions may have been lost from platelets that were functionally defective but still morphologically intact, or these changes may simply represent a slow equilibrium reaction of the chromate between the blood elements.

TABLE II. RECOVERY OF PLATELETS AND RADIOACTIVITY IN VARIOUS FRACTIONS FOLLOWING STORAGE OF RADIOCHROMATE-TAGGED PLATELETS IN A SUSPENSION OF RED CELLS IN PLASMA (MEAN AND RANGE OF PER CENT RECOVERY IN 6 EXPERIMENTS FOR EACH PERIOD)

	PER CENT RECOVERY OF PLATELETS AND RADIOACTIVITY					
	18 HOURS		44 HOURS		68 HOURS	
FRACTIONS	PLATELETS	RADIOACTIVITY	PLATELETS	RADIOACTIVITY	PLATELETS	RADIOACTIVITY
Platelets in supernatant plasma	95.6 (74.0-111.0)	67.2 (61.1-73.1)	73.0 (48.0-98.0)	57.7 (47.4-68.9)	71.3 (40.0-104.0)	47.1 (38.0-55.0)
Platelets in 2 saline washes of red cell fraction	14.3 (9.0-17.3)	12.9 (10.3-16.0)	25.1 (9.0-42.0)	16.3 (12.8-18.6)	20.9 (15.0-30.0)	19.4 (19.0-20.0)
Plasma after centrifugation	—	8.8 (4.8-12.6)	—	8.3 (6.5-11.9)	—	9.5 (6.8-13.7)
Saline washes after centrifugation	—	3.2 (2.4-4.0)	—	3.2 (2.0-4.9)	—	5.4 (4.1-8.0)
Red cells	1.2 (0-2.4)	4.7 (3.4-6.0)	1.6 (0-3.2)	10.4 (3.2-14.4)	2.1 (0.8-2.7)	17.6 (12.4-23.3)
Total	111.1	95.8	99.7	95.9	94.3	99.0

2. *In Vivo Studies. Normal Rabbits.*—In 9 normal animals studied for as long as 120 hours, the mean number of total platelets injected was 2.7×10^{10}, the mean total radioactivity of injected platelets was 21,120 counts per second, and the mean total radioactivity of injected plasma was 21,200 counts per second. Total radioactivity injected varied from 11 to 37 microcuries. Radioactivity persisting in platelet, plasma, and red cell fractions of 20 ml. blood samples at varying time intervals after injection of labeled platelets in these 9 rabbits is shown in Table III and Fig. 1. On the basis of an estimated blood volume of 6 per cent of body weight[10, 11] the mean percentage of total radioactivity injected in plasma and platelets that persisted at 2 hours in each of the 3 fractions was calculated* to be as follows: platelets 11.5, plasma 3.1, and red cells 24.6. Since

*$\frac{\text{c.p.s. in fraction of 20 ml. samples} \times 100}{\text{total injected c.p.s.}} \times \frac{\text{body weight} \times 0.06}{20}$.

TABLE III. RADIOACTIVITY (COUNTS PER SECOND) IN PLATELET, PLASMA, AND RED CELL FRACTIONS OF 20 ML. BLOOD SAMPLES AT VARYING TIME INTERVALS AFTER INJECTION OF CHROMATE-TAGGED PLATELETS INTO 9 RABBITS

2 HOURS			16-20 HOURS			43-48 HOURS			67-69 HOURS			92-94 HOURS			116-120 HOURS		
PLATE-LETS	PLASMA	RED CELLS	PLATE-LETS	PLASMA	RED CELLS	PLATE-LETS	PLASMA	RED CELLS	PLATE-LETS	PLASMA	RED CELLS	PLATE-LETS	PLASMA	RED CELLS	PLATE-LETS	PLASMA	RED CELLS
			313	96	199	99	48	152	29	42	89	5	52	34			
			90	16	407	22	23	298	5	39	213				5	15	203
650	153	800	268	55	912	93	41	383				5	30	226	5	43	125
536	136	2,020	249	48	922	102	36	538	39	49	628	8	55	249			
907	123	1,585	454	45	942	248	28	581	58	40	348				5	43	247
482	117	1,230	254	62	474	79	0	516	32	52	377	5	26	230			
392	441	1,058	226	0	806	61	63	435	18	0	256				6	27	155
451	61	1,078	309	42	504	106	32	503	38	24	310	9	27	264			
1,695	157	3,690	724	55	1,860	300	49	1,287	88	55	1,080				6	44	878

half of the injected radioactivity was in platelets, an average of 23 per cent of the tagged platelets can be assumed to be circulating 2 hours after injection. Platelet radioactivity decreased progressively, significant amounts persisting through the third day (66 to 69 hours). (As shown in Table II not all of the tagged platelets contained in blood samples are recovered in the platelet fraction, 14 to 25 per cent being trapped with the red cells. The number of tagged platelets surviving in vivo, therefore, was greater, and the red cell radioactivity

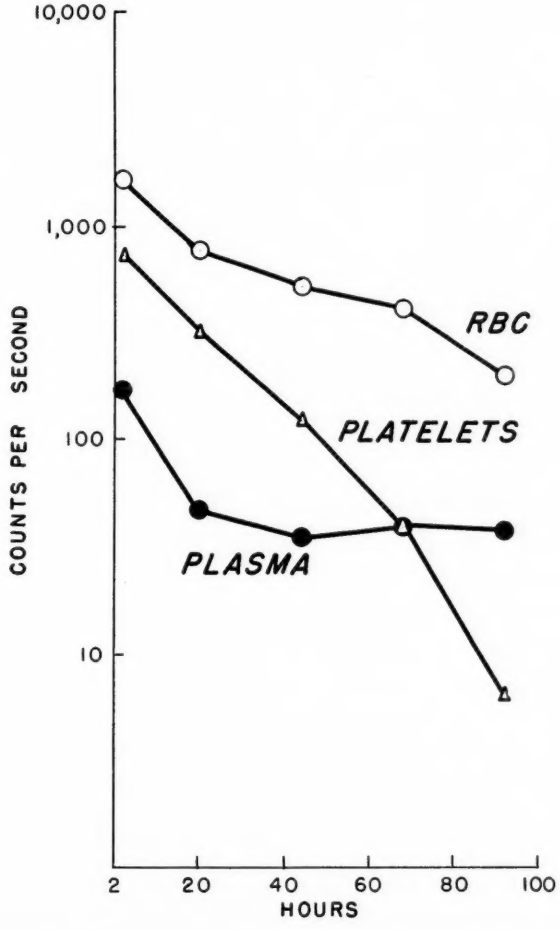

Fig. 1.—Mean radioactivity in counts per second recovered from various fractions of 20 ml. cardiac blood at intervals after the injection of tagged platelets into 9 rabbits (plotted on semilogarithmic scale).

less, than is apparent from the data in Table III. The effect of repeated removal of up to 20 c.c. of blood must also be considered in evaluating the significance of persisting platelet radioactivity. In addition to the actual removal of a portion of the labeled platelets from the circulation, the resulting expansion of plasma to offset the blood loss will dilute the remaining platelet radioactivity. These changes, while not affecting the validity of the observations on radioactive

platelet survival, would shorten the period in which enough labeled platelets to measure were still circulating, and would not apply in man.)

Inspection of Table III reveals a preponderance of activity in the red cell fraction after 48 hours. The significance of the platelet radioactivity in these and later samples is more apparent if one considers that the volume of the platelet fraction is less than 1 per cent of the volume of either the red cell or plasma fractions.

Animals Receiving Serum.—In animals subsequently injected with serum, the mean total injected radioactivity was 12,200 and 11,900 counts per second in

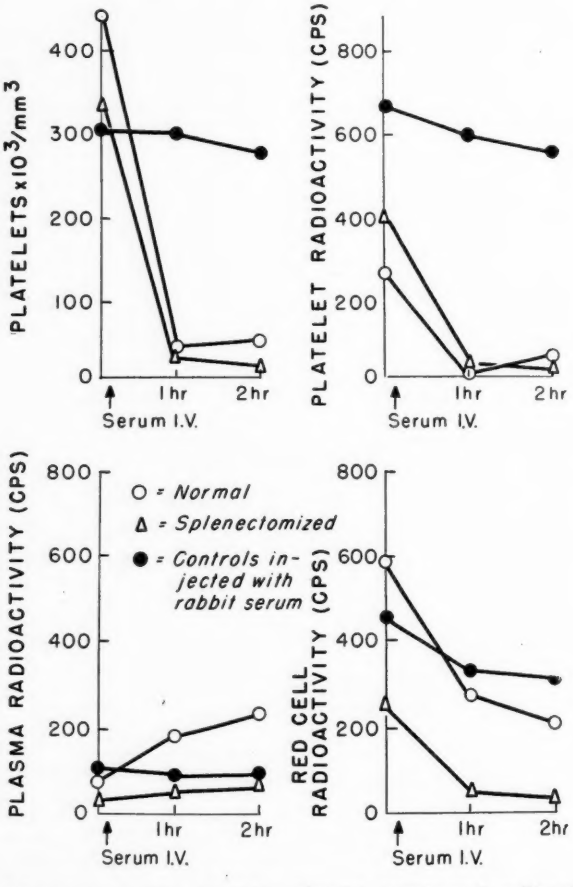

Fig. 2.—Effect of intravenous serum on mean platelet counts and radioactivity and on mean plasma and red cell radioactivity of 20 ml. blood samples.

platelets and plasma, respectively. Table IV and Fig. 2 show the mean platelet counts and radioactivity in counts per second recovered in the various blood fractions of experimental and control groups of animals. In normal rabbits injected with human serum there was a marked decrease in the platelet count which persisted throughout the experiment. There was a parallel fall in recoverable platelet radioactivity from a mean preinjection value of 275 counts per second to 18 counts per second 1 hour after serum injection. Splenectomized animals injected with human serum showed similar changes in platelet count

and platelet radioactivity. In normal control animals injected with rabbit serum, there was no drop in platelet count and no acceleration of the normal rate of disappearance of platelet radioactivity.

TABLE IV. EFFECT OF INTRAVENOUS HUMAN SERUM ON RABBIT PLATELET COUNTS AND PLATELET FRACTION RADIOACTIVITY OF 20 ML. BLOOD
(Mean values and range for each group)

	BEFORE INJECTION	30-60 MINUTES	90-180 MINUTES
Normal rabbits (10)			
Platelets × 10^3/mm.^{3}	443 (272-630)	35 (5-140)	42 (12-90)
Radioactivity in counts per second	275 (104-861)	18 (3-37)	46 (9-74)
Splenectomized rabbits (5)			
Platelets × 10^3/mm.^{3}	339 (288-402)	23 (14-30)	14 (10-18)
Radioactivity in counts per second	393 (305-474)	25 (3-59)	18 (1-37)
Controls injected with rabbit serum (5)			
Platelets × 10^3/mm.^{3}	306 (230-400)	300 (222-400)	275 (196-396)
Radioactivity in counts per second	667 (420-836)	599 (375-783)	561 (353-722)

In animals injected with human serum there was a fall in red cell radioactivity and a small rise in plasma radioactivity. A fall in hematocrit was also noted (data not presented).

Table V gives the mean concentration of radioactivity in $\frac{\text{counts per second/gram of tissue} \times 100}{\text{injected counts per second}}$ recovered in spleens, livers, and lungs of rabbits injected with tagged platelets, chromate^{51} in plasma, and chromate^{51}-tagged red blood cells. In animals injected with tagged platelets there was an appreciable concentration of radioactivity in the spleen, and a slight and inconstant concentration in liver and lung. In animals injected with either chromate in plasma or chromate-tagged red cells no splenic concentration of radioactivity was demonstrated. All other organs assayed contained no significant amounts of radioactivity for any group. No appreciable differences in splenic concentration of radioactivity were observed between animals rendered thrombocytopenic with human serum and control animals.

TABLE V. MEAN CONCENTRATION AND RANGES OF RADIOACTIVITY EXPRESSED IN $\frac{\text{COUNTS PER SECOND/GRAM OF TISSUE} \times 100}{\text{INJECTED COUNTS PER SECOND}}$ IN VISCERA OF RABBITS INJECTED WITH (A) CHROMATE-TAGGED PLATELETS AND HUMAN SERUM, (B) CHROMATE-TAGGED RED CELLS, AND (C) CHROMATE IN PLASMA

	SPLEEN	LIVER	LUNG
A. Tagged Platelets and serum			
Normal (10 animals)	3.1 (1.2-8.0)	0.2 (0-0.4)	0.1 (0-0.3)
Splenectomized (5 animals)	—	0.3 (0.3-0.5)	0.0
Controls (5 animals) injected with rabbit serum	5.5 (3.7-7.4)	0.2 (0.1-0.4)	0.2 (0-0.5)
B. Tagged Red Cells (5 animals)	0.3 (0.0-0.6)	0.02 (0.0-0.1)	0.2 (0.0-0.8)
C. Chromate in Plasma (3 animals)	0.0	0.0	0.0

DISCUSSION

In previous attempts to label platelets in vitro with P^{32} (ref. 6), and I^{131} (ref. 7), no measurable numbers of tagged platelets survived on reinjection. With radiophosphorus the duration (16 to 18 hours) and the trauma of the labeling process presumably damaged the platelets. Labeling with radioiodine was rapid, but could not be done in the presence of plasma because of the affinity of plasma proteins for iodine. Separation from plasma and saline washing of platelets before labeling with I^{131} were excessively traumatic as indicated by loss of ability of tagged platelets to promote clot retraction. Robertson, Milne, and Cohn[8] reported the successful labeling of rat platelets in vitro with Cr^{51} in the form of chromic chloride, but found that there was such a rapid loss of radioactivity from the labeled platelets after reinjection that the method was not suitable for measuring the life span of platelets in the circulation. This is not surprising in view of the high affinity of plasma proteins for chromic ion as reported by Visek and associates.[12]

The radiochromate method seems to surmount disadvantages of previous labeling methods because the total labeling process occupies less than 5 hours and can be carried out in the presence of plasma. There are some disadvantages inherent in the method, however. Since repeated washings of platelets are accompanied by diminishing ability to promote clot retraction, a relatively large amount of unbound chromate in the plasma containing the labeled platelets was injected. This radiochromate was taken up by red cells in large part but did not interfere with the subsequent detection of platelet radioactivity provided that a careful separation of platelets from red cells and plasma was performed. From 5 to 18 per cent of radiochromate was lost from platelets to red cells in vitro and this exchange may be presumed to occur to some extent in vivo. There is some unavoidable damage to platelets during the labeling as evidenced by rapid disappearance of more than half of the labeled platelets after reinjection. The 3-day recoverability period of the labeled platelets may well be due to the removal of repeated samples of blood from the rabbits rather than a shortened life span of the labeled platelets.

Evidence that the radioactivity in the platelet fraction in all these experiments is associated with functioning platelets is summarized as follows: (1) Tagged platelets retained the ability to promote clot retraction. (2) Tagged platelets stored with red cells retained most of their radioactivity. (3) Significant concentrations of radioactivity persisted in the platelet fraction of blood up to the third day, and measurable amounts up to the fourth day. (4) In animals injected with unbound radiochromate in plasma there was no significant radioactivity in the platelet fraction of subsequent blood samples. (5) The parallel fall in platelet count and platelet radioactivity measurements during human serum-induced thrombocytopenia indicates that the platelets labeled with radiochromate were subject to the platelet depressing effect of serum to the same degree as unlabeled platelets. (6) Although a concentration of radioactivity was found in spleens of animals injected with tagged platelets, no splenic concentration was found in animals injected with either chromate alone or chromate-tagged red cells.

In animals injected with human serum the fall in red cell radioactivity can be ascribed to the hemolytic effect of injected heterologous serum. The increase in plasma radioactivity observed in the animals injected with serum is what might be expected from radiochromate released from destroyed platelets and red cells.

That the spleen is not responsible for the thrombocytopenia induced by heterologous serum injection is indicated by (1) rapid decrease of platelets and platelet radioactivity in splenectomized animals and (2) similar concentrations of radioactivity in the spleens of animals injected with heterologous and homologous serum. The concentration of radioactivity in the spleen in animals injected with labeled platelets suggested that this organ was an important site of destruction for tagged platelets.

CONCLUSIONS

1. Rabbit platelets labeled in vitro with sodium chromate^{51} survived on reinjection.

2. Significant amounts of platelet radioactivity were recovered from the circulation for 3 days.

3. Evidence is offered that the labeled platelets retain their function.

4. Chromate^{51} tagging affords a tool of value in the study of the life span of platelets in the rabbit.

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STUDIES ON PLATELET ANTIGENS

II. A_1 AND A_2 SUBGROUPS IN BLOOD PLATELETS

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IN PREVIOUS publications,[1-3] we have reported the existence of A, B, O, and AB groups in blood platelets corresponding to these groups in red blood cells.

The identity of the A and B antigens in platelets and red blood cells has been proved by: (1) platelet agglutination with antisera; (2) cross-adsorption of isoagglutinins with thrombocytes and erythrocytes; (3) elution of isoagglutinins adsorbed by platelets; (4) inhibition of isoagglutinins with saliva from secretors; and (5) neutralization of antisera with A and B group specific substances.

The agglutination of platelets by A and B antisera has been confirmed by Ruggieri and Bolognesi[4] and more recently by Dausset.[5] Adsorption of A and B isoagglutinins with thrombocytes was also reported by Moureau and André.[6, 7] The latter authors used crushed platelets for their adsorption experiments.

The present study shows that blood platelets of Group A may be divided into the subgroups A_1 and A_2, similar to those in erythrocytes.

MATERIALS AND METHODS

Details of methods used in studies of platelet antigens have been reported.[1-3] In view of certain slight modifications, a short account is given.

All glassware was siliconized before use. Disodium Sequestrene (disodium ethylenediaminetetraacetate dihydrate) was employed as the anticoagulant.

Triton W.R. 1339 was used to prevent irreversible clumping of the platelets caused by centrifugation.

A solution composed of disodium Sequestrene 1 per cent, and Triton W.R. 1339 1 per cent was prepared in sodium chloride 0.7 per cent. One milliliter of this solution was added to 9 ml. of blood.

Preparation of the Platelet Suspension.—Blood was taken from healthy normal persons in a siliconized syringe containing the disodium Sequestrene-Triton solution. The blood was immediately transferred into a large centrifuge tube with a flat bottom (a conical bottom made the resuspension of platelets difficult), and was centrifuged in a horizontal centrifuge for five to seven minutes at 800 r.p.m. The plasma was pipetted off and centrifuged for a further five to seven minutes at 800 r.p.m. in order to remove the remaining red and white blood cells. The supernatant plasma, containing a pure suspension of

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thrombocytes, was next centrifuged for ten minutes at 2,000 r.p.m. to obtain a sediment of blood platelets. The plasma was siphoned off and a white pasty mass of platelets remained on the bottom of the tube.

To a platelet sediment prepared from 40 to 50 ml. of blood, 0.5 ml. of normal saline was added, and the tube was shaken gently for 3 to 5 minutes at about 6° C., to obtain a homogeneous concentrate of platelets.

Preparation of the Sera.—Blood was taken from healthy donors under sterile precautions. Sera were inactivated at 56° C. for thirty minutes. Only clear and uncontaminated sera were used. They were titrated for anti-A and anti-B isoagglutinins by the double serial dilution method against freshly prepared red blood cell suspensions. Sera were stored at -20° C. until used.

Technique of Isoagglutinin Adsorption by Platelets.—To a platelet sediment from 40 to 50 ml. of blood, prepared as described above, 0.7 ml. of a high titer antiserum was added. The platelet serum mixture was left for 15 to 20 hours at 6° C., being occasionally shaken. It was then centrifuged for 10 minutes at 2,000 r.p.m. and the supernatant pipetted off. The adsorbed serum was now examined for its isoagglutinin titer against red blood cells.

Preparation of Eluates.—Platelet sediments used in adsorption experiments were washed thrice in large volumes of normal saline to ensure complete removal of serum. After the last wash the supernatant saline was removed and the platelet sediment resuspended in 0.4 ml. of saline. The resuspended platelets were then agitated for 5 to 7 minutes in a 56° C. water bath, centrifuged at 3,000 r.p.m. for 1 minute in cups jacketed with water at 56° C., and the eluate immediately removed. Eluates were stored frozen at -20° C. until used.

EXPERIMENTAL

Agglutination of Platelets.—To a platelet concentrate, normal saline was added in order to obtain a platelet suspension of about 1 million cells per cubic millimeter.

To 0.5 ml. of test serum in a siliconized tube was added 0.1 to 0.2 ml. of the platelet suspension. The tube was gently shaken to obtain a homogeneous mixture and left in the refrigerator at 6° C. for 3 hours.

Before the results were read, the tubes were again gently shaken and examined macroscopically. One drop of the serum-platelet mixture was then withdrawn by a capillary pipette and examined microscopically at low magnification.

In all platelet samples belonging to the A_1 group there was a clear-cut agglutination after 3 hours. After 6 hours the agglutinates were larger and the number of the non-agglutinated platelets in a microscopic field was small. At this time the agglutination could easily be read macroscopically.

In all A_2 platelet specimens, agglutination proceeded much more slowly and no agglutinates could be observed microscopically even after 6 hours. After 9 hours only small agglutinates could be observed, similar to those seen after 2 to 3 hours with A_1 samples.

The agglutination of both A_1 and A_2 platelet samples was carried out in B and O sera with titers of at least 1:64 anti-A_1 and 1:32 anti-A_2. It was interesting to note, however, that O sera with anti-A_1 and anti-A_2 titers, similar to those of B sera, agglutinated A_2 platelets within 3 hours.

Sixty agglutination tests with samples of A_1 and A_2 platelets against different B and O sera were performed.

Adsorption of Isoagglutinins by Platelets of Subgroups A_1 and A_2.—In 20 adsorption experiments of B sera performed with A_1 thrombocytes the titer of the adsorbed serum was reduced from about 1:128 to 1:2. A_1 platelets markedly reduced both the anti-A_1 and the anti-A_2 titers of the examined B sera.

In 5 adsorption experiments of B sera performed with A_2 thrombocytes the titer was reduced from 1:64 to 1:32 only. A_2 platelets were more active in the adsorption of anti-A_2 than of anti-A_1 isoagglutinins.

TABLE I. ADSORPTION OF B SERUM WITH A_1 AND A_2 PLATELETS

		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Anti-A_1 and -A_2 titers before adsorption	A_1	+++	+++	+++	+++	+++	+++	++	±
	A_2	+++	+++	+++	+++	++	+	-	-
Titers after one adsorption with A_1 platelets	A_1	++	+	-	-	-	-	-	-
	A_2	+	-	-	-	-	-	-	-
Titers after one adsorption with A_2 platelets	A_1	+++	+++	+++	+++	+++	+++	+	-
	A_2	+++	+++	+++	++	+	-	-	-

Typical results of adsorption experiments with A_1 and A_2 platelets are summarized in Table I.

Elution of Isoagglutinins Adsorbed by Platelets.—Eluates from the platelets which were used in the adsorption experiments were titrated against A_1 and A_2 erythrocytes. Eluates prepared from A_1 platelets had a lower titer against both A_1 and A_2 red blood cells than the eluates prepared from the A_2 platelets.

Table II shows typical elution results. All comparative experiments were done simultaneously and under identical conditions.

TABLE II. TITERS OF ELUATES FROM A_1 AND A_2 PLATELETS

		1:1	1:2	1:4	1:8	1:16	1:32	1:64
Eluates from A_1 platelets titrated with:	A_1 erythrocytes	+++	+++	++	+	-	-	-
	A_2 erythrocytes	++	+	-	-	-	-	-
Eluates from A_2 platelets titrated with:	A_1 erythrocytes	+++	+++	+++	++	+	-	-
	A_2 erythrocytes	+++	+++	+++	++	++	+	-

COMMENT

In accordance with previous results on the existence of A and B antigens in platelets, it has been established that A_1 and A_2 subgroup antigens can be distinguished in platelets comparable to those in red blood cells.

Results of agglutination of A_1 platelets are similar to those of the previous studies.[1, 2] Potent B sera of a titer not less than 1:16 gave distinct agglutination of platelets after 3 hours. When the same B sera were examined against A_2 platelets, agglutination appeared more slowly, so that microscopic results could be read only after 9 hours' incubation at 6° C. At this time the degree of agglutination of A_2 platelets was similar to that obtained with A_1 platelets after 3 hours.

Results of agglutination of A_2 platelets by B and O sera were compared. It was found that O sera agglutinated A_2 platelets within 3 hours, while B sera gave distinct agglutination of the same samples of A_2 platelets only after 9 hours. When O sera were used, the agglutinates were larger and the percentage of unagglutinated platelets in a microscopic field was smaller as compared with results of agglutination by B sera.

Adsorption of high-titered B sera with A_1 platelets reduced significantly the titers of both anti-A_1 and anti-A_2 isoagglutinins. Adsorption of the same sera with A_2 platelets reduced anti-A_1 and anti-A_2 isoagglutinins only slightly.

Eluates from A_2 platelets gave a significantly higher anti-A titer than eluates prepared from A_1 platelets, when examined against both A_1 and A_2 erythrocytes.

The afore-mentioned results of agglutination, adsorption, and elution experiments are very similar to those usually obtained in experiments with A_2 erythrocytes. A_2 erythrocytes give only weak reactions with potent B sera and are usually agglutinated more markedly by O sera. The adsorption avidity of A_2 erythrocytes toward the anti-A agglutinin is not as great as that of A_1 erythrocytes.

Elution experiments with A_2 erythrocytes show that it is easier to separate adsorbed isoagglutinins from A_2 erythrocytes than from A_1 cells.[8]

SUMMARY

The existence of antigenic subgroups A_1 and A_2 in blood platelets similar to these subgroups in red blood cells has been established.

Results of agglutination, adsorption, and elution experiments with A_1 and A_2 platelets have been compared.

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A STUDY OF THE EFFECT OF DISODIUM CALCIUM VERSENATE (Ca EDTA) ON IRON EXCRETION IN MAN*

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INTRODUCTION

WITHIN the past few years one of the aminopolycarboxylic acids, ethylenediamine tetraacetic acid (EDTA), has been reported to be useful in the therapy of poisoning with lead, mercury, and certain radioactive elements.[1] The disodium calcium salt of EDTA forms strong water-soluble chelates with several metals at physiologic pH's. It is essentially nonmetabolizable and owes its low toxicity to the fact that in solution it does not decrease the calcium ion concentration of body fluids even when injected rapidly. Foreman[2] found that "Fe–3 Specific," a related chelating agent, increased the excretion of Fe^{59} by a factor of 2 to 2.5 in rats. Wishinsky and associates[3] have used Ca EDTA intravenously in one case of hemochromatosis with the intent of increasing iron excretion. A similar study has been described by Rubin and co-workers,[4] who administered Versenol† to two patients with suspected hemochromatosis. Versenol is another member of the chelating group of compounds with greater affinity for iron than Ca EDTA.

In neither the case of Ca EDTA nor Versenol has it been demonstrated that the increases in iron excretion were more than a small fraction of the amount removed by a single phlebotomy, and it is doubtful if this form of chemotherapy can compete with bleeding as a means of mobilizing iron.

Prior to the publication of the reports of Wishinsky[3] and Rubin,[4] a study of the intravenous and oral use of Ca EDTA was initiated in a series of patients with normal amounts of body iron as well as a group with hemochromatosis. The data accumulated in this investigation constitute the subject of this report.

MATERIAL AND METHODS

The clinical material includes 15 male subjects between the ages of 25 and 75 (Table I). Two of the patients with primary hemochromatosis (E. J. R. and A.M.) had previously been treated by phlebotomy, E. J. R. for two years, with the removal of over 50 pints of blood, and A. M. for one year, with the removal of 21 pints of blood. During the course of the investigation Patients A. M. (primary hemochromatosis) and C. K. (hypogonadism) were placed on iron balance while the others were kept on a regular diet.

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†N-hydroxyethylethylenediamine triacetic acid.

TABLE I. CLINICAL MATERIAL

SUBJECT	AGE	CLINICAL DIAGNOSIS	LIVER BIOPSY
E. J. R.	56	Primary hemochromatosis	Positive
E. M.	64	Primary hemochromatosis	Positive
A. M.	60	Primary hemochromatosis	Positive
G. W. B.	62	Primary hemochromatosis	Positive
F. N.	62	Primary hemochromatosis	Positive
F. D.	62	Primary hemochromatosis	Positive
H. S.	47	Rheumatoid and hypertrophic arthritis with obesity and fatty liver	Biopsy performed
J. M. W.	25	Aplastic anemia; hemochromatosis secondary to transfusions	No biopsy
M. M.	63	Gastrectomy for leiomyosarcoma	No biopsy
C. K.	48	Postpuberal hypogonadism	No biopsy
J. A.	44	Duodenal ulcer—on therapy	No biopsy
J. T.	75	Pernicious anemia—in remission	No biopsy
F. O.	58	Convalescent alcoholic polyneuropathy	No biopsy
R. B.	60	Normal control	No biopsy
C. A.	48	Normal control	No biopsy

Ca EDTA* was administered intravenously to all patients and, in addition, 4 of the group (C. K., E. J. R., A. M. and M. M.) received courses of oral medication. When given intravenously a 1 per cent solution of the drug was infused slowly, either in normal saline or 5 per cent glucose, at a rate of 1 Gm. every forty-five to sixty minutes. As a rule, the amount to be given in one day was divided into two doses when the total dose exceeded 4 Gm. One-half was administered in the morning, and the other half in the afternoon. When taken orally, 250 mg. tablets of Ca EDTA were administered at two-hour intervals and the dosage schedule so arranged that medication coincided with meals.

Iron was determined on samples of diet, feces, and urine ashed at 500° C. in a muffle furnace, and in some cases on ashed serum by modification of the orthophenanthroline method of Hummel and Willard.[5] Fifty per cent ammonium acetate was used for the buffer in place of sodium acetate; the sequence of adding reagents was in the order recommended by Bandemer and Schaible.[6] The analysis of unashed serum for iron followed the procedure of Schales,[7] while the unsaturated iron-binding capacity of serum (UIBC) was performed as directed by Cartwright and Wintrobe.[8] These latter analyses, as well as complete blood counts, were made at weekly intervals. The maximal absorption of the orthophenanthroline iron complex was determined on the DU model Beckman spectrophotometer and found to be 514 mμ. Colorimetry was performed using this instrument.

Special precaution was taken in the preparation of iron-free water for all reagents by running distilled water through a mixed column of strong ion exchange resins (IR-120, the acid form, and Amberlite IRA-400, the hydroxide form). Water, reagents, and urine specimens were stored in polyethylene bottles. Glassware was cleaned with a 2 per cent solution of a chelating agent† and rinsed in deionized water four times.

RESULTS

Intravenous Ca EDTA.—

A. *In patients without hemochromatosis:* Fig. 1 reveals the effect of the intravenous administration of Ca EDTA on urinary iron in a group of 8 patients who served as controls. None of these excreted iron in the urine in excess of 3 mg. in twenty-four hours. This was in contrast to the group of 7 patients with hemochromatosis who received comparable doses of the drug (1 to 4 Gm.

*Disodium calcium EDTA was obtained from Dr. Norman W. Karr, Director, Clinical Research, through the courtesy of the Riker Laboratories, Inc., Los Angeles, Calif.

†Versene Fe-3 Specific, obtained from Bersworth Chemical Company, Framingham, Mass.

daily) and 5 of whom excreted more than 3 mg. of urinary iron during a twenty-four-hour period. That the generally lower concentration of serum iron was not the only factor in the smaller urinary iron excretion of the control group is suggested by the following observations: Patient M. M. (Fig. 1) who had received 15 transfusions following gastrectomy for leiomyosarcoma one year previously had a concentration of serum iron of 175 μg per cent which

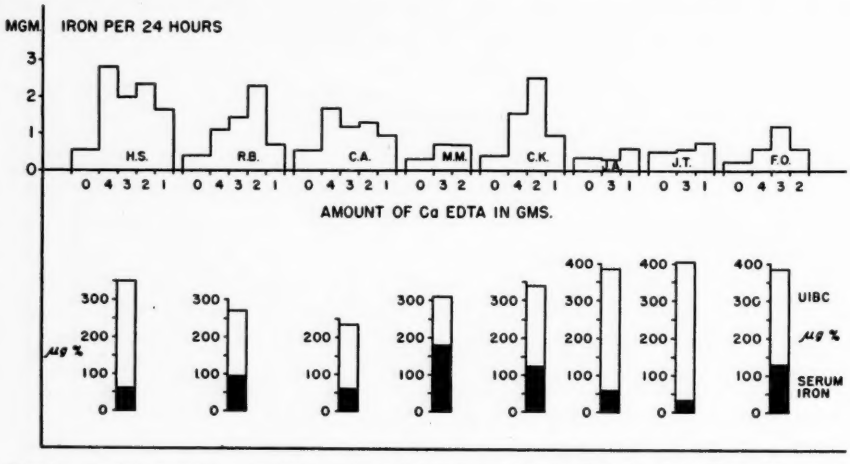

Fig. 1.—Urinary iron excretion with intravenous Ca EDTA in control subjects.

Patient	*Diagnosis*
H. S.	Rheumatoid and hypertrophic arthritis with moderate obesity and fatty liver
R. B.	Normal control
C. A.	Normal control
M. M.	Gastrectomy for leiomyosarcoma
C. K.	Postpuberal hypogonadism
J. A.	Duodenal ulcer—on therapy
J. T.	Pernicious anemia—in remission
F. O.	Convalescent alcoholic polyneuropathy

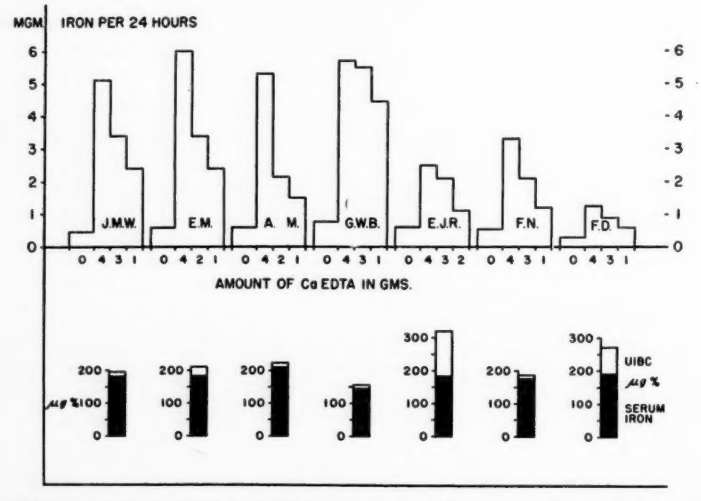

Fig. 2.—Urinary iron excretion with intravenous Ca EDTA in primary and secondary hemochromatosis. Patient J. M. W. had hemochromatosis secondary to aplastic anemia and multiple transfusions. All others had primary hemochromatosis. Patient E. J. R. has had 50 pints of blood removed in the past two years.

was within the range of values found in the patients with hemochromatosis. The excretion of iron in the urine was very little affected by an infusion of 3 Gm. of the drug. Patient H. S., on the other hand, an individual with rheumatoid and hypertrophic arthritis, obesity, and a fatty liver, with a serum iron of 57 μg per cent excreted more iron in the urine after Ca EDTA than any other subject in this group.

B. *In patients with hemochromatosis:* The intravenous injection of a solution of Ca EDTA regularly produced a substantial, although variable, increase in the urinary excretion of iron in the 7 cases of hemochromatosis (Fig. 2 and Table II). Maximal responses of eight- to tenfold the amount in control urines were observed following the infusion of 4 Gm. of the drug. However, it should be pointed out that quantitatively the iron eliminated was small in amount, an average of 3 to 6 mg. in twenty-four hours. In 2 subjects (E. J. R. and A. M.) in whom urine was collected at six-hour intervals, it was found that the main effect occurred within the six-hour period immediately following the injection of Ca EDTA. It should be noted, too, that the output of urinary iron during intravenous therapy (Figs. 3 and 5) was more marked during the first and second days and that continued administration of the chelating agent in equal or slightly larger amounts during successive days was associated with a decrease in the excretion of urinary iron from the peak value, but still appreciably above that of the control level. Increasing the dose of Ca EDTA from 5 Gm. the first day to 10 Gm. on the fifth day (Patient A. M., Fig. 3) resulted in only a slight increase in urinary iron from 5 mg. to 5.7 mg. Some elevation of urinary iron persisted for several days after the drug was stopped.

TABLE II. PRIMARY AND SECONDARY HEMOCHROMATOSIS

	IRON EXCRETED IN URINE IN 24 HR., MG.							
	Ca-EDTA, I.V., GM.					S.I.†	U.I.B.C.‡	T.I.B.C.§
PATIENT	1	2	3	4	CONTROL	μG PER CENT		
J. M. W.	2.4		3.4	5.1	0.467(4)*	189	0.0	189.0
E. M.	2.4	3.4	8.5	6.0	0.621(13)	183	25.0	208.0
F. N.	1.2	1.75	2.1	3.29	0.530(8)	173	12.5	185.5
G. W. B.	4.45		5.5	5.7	0.735(13)	144	0.0	144.0
E. J. R.		1.16	2.1	2.5	0.61 (13)	182	140.0	322.0
A. M.	1.5	2.15		5.1	0.593(9)	210	0.0	210.0
F. D.	0.600	0.65	0.9	1.2	0.266(9)	190	80.0	270.0

*Number of determinations averaged.
†S.I.=serum iron.
‡U.I.B.C.=unsaturated iron-binding capacity.
§T.I.B.C.=total iron-binding capacity.

The comparisons between oral and intravenous therapy were made while the patient was ingesting a constant diet in order that an iron balance could be kept (Fig. 4). The average intake of food iron was 22 mg. per day and, during a ten-day control period, the retention of iron was 2 to 3 mg. daily. In the subsequent five days (Period 3), 2 Gm. of the chelating agent was given intravenously every morning without appreciable effect on the iron balance but producing a moderate increase in urinary iron. During the following control period (Period 4), the fecal iron increased to equal the intake. This may have

been due to blood swallowed by the patient subsequent to the extraction of a tooth. The oral ingestion of 40 Gm. of Ca EDTA in a dosage increasing from 2 to 9 Gm. daily (Periods 6 and 7) had no appreciable effect on urinary iron or iron balance. When a five-day course of the drug, also totaling 40 Gm., was given intravenously in a dose that increased from 5 to 10 Gm. daily, there was the usual pronounced effect on urinary iron which rose from approximately 0.5 mg. (pretreatment) to 6 mg. in twenty-four hours. In this instance, the iron balance became slightly negative due to the increased excretion of iron in the urine. In

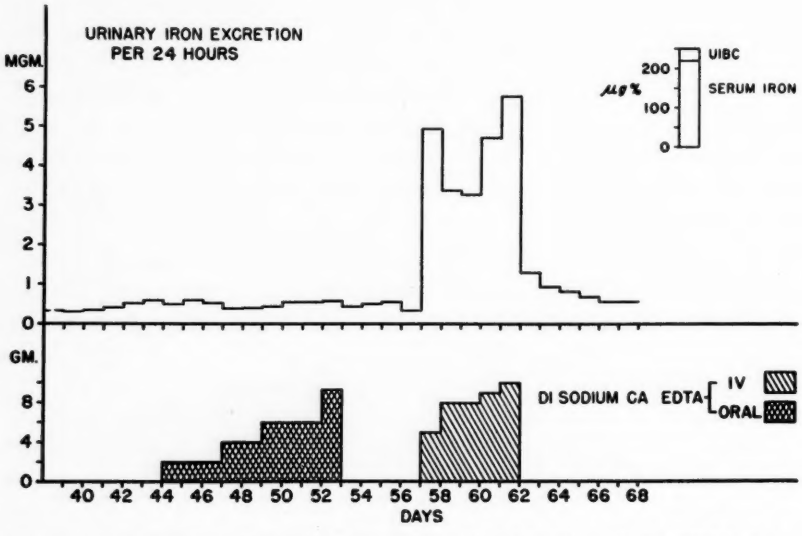

Fig. 3.—Chart comparing the oral and intravenous administration of Ca EDTA in a patient (A. M.) with primary hemochromatosis.

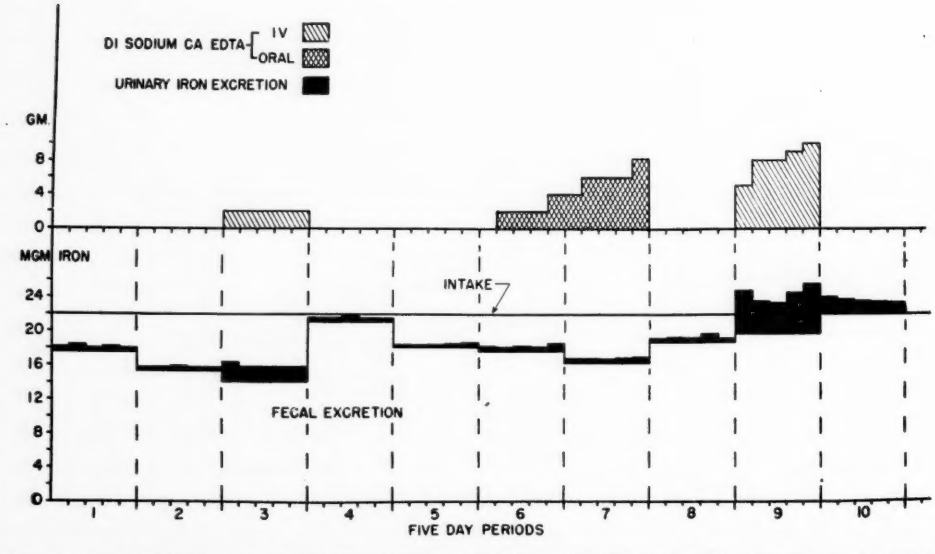

Fig. 4.—Iron balance data on same patient as in Fig. 3 during oral and intravenous Ca EDTA administration. During Period 4 patient had a tooth root extraction.

the control period which followed (Period 10) fecal iron again reached a peak value which exceeded the intake. In this case, there was no reason to suspect bleeding into the gastrointestinal tract and, hence, the high fecal iron cannot be explained on this basis.

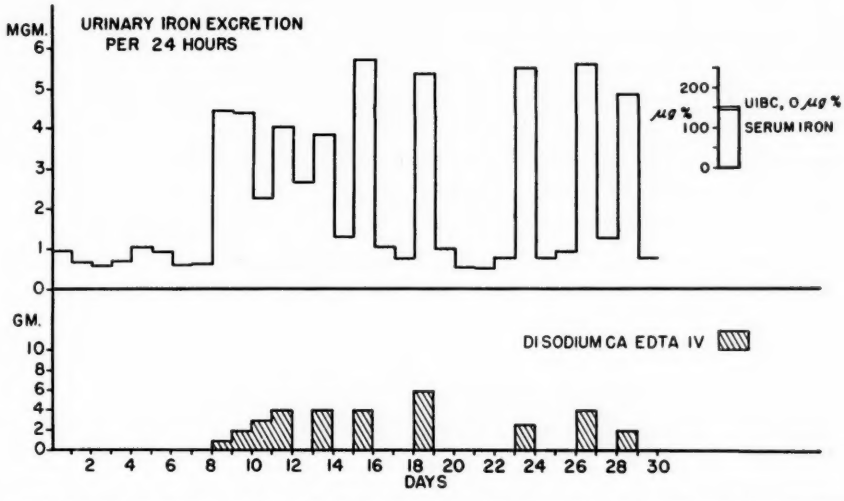

Fig. 5.—Effect of intravenous Ca EDTA on urinary iron excretion in primary hemochromatosis (Patient G. B.), comparing consecutive with intermittent administration.

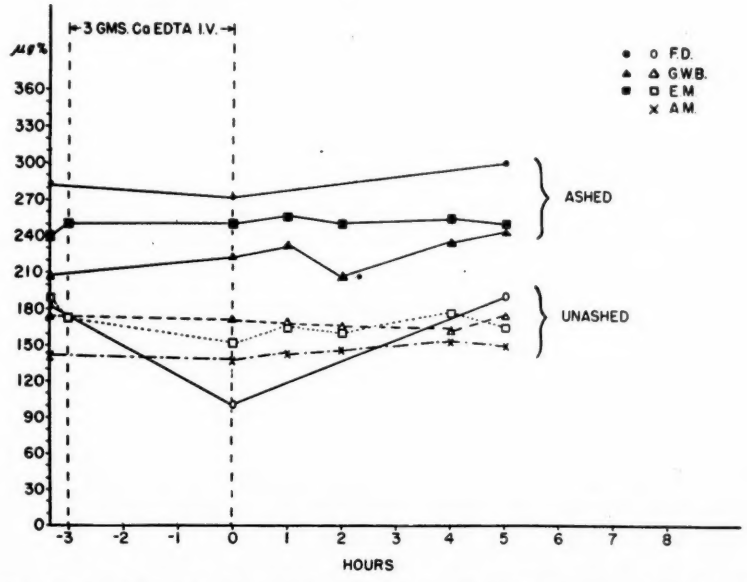

Fig. 6.—Effect of i.v. Ca EDTA on serum iron in 4 cases with hemochromatosis. Ashed values on Patient A. M. are as follows: 138, 148, 156, 171, 177, and 175 μg per cent.

C. *Effect on serum iron:* Serial determinations of serum iron using both ashed and unashed serum were made in 4 patients with primary hemochromatosis, following the infusion of 3 Gm. of Ca EDTA (Fig. 6). In 3 of

the 4 patients there was no appreciable reduction in the serum iron either in the ashed or unashed sample. In the fourth subject (F. D.), the serum iron fell from 180 μg per cent to 100 μg per cent at the end of the infusion; the ashed serum on this subject failed to show this decrease. In general, ashed serum specimens yielded higher values than unashed specimens. Although the number of cases is small, it appears that Ca EDTA does not reduce the serum iron.

DISCUSSION

The effect of parenteral administration of the calcium salt of ethylenediamine tetraacetic acid to patients with normal concentrations of serum iron and those with hemochromatosis indicates the formation of a soluble iron complex. In nearly every instance, the slow infusion of 2 Gm. or more of the drug dissolved in physiologic salt solution led to an appreciable increase in urinary iron. Most of the iron was excreted within a period of six hours following the infusion, an interval of time which presumably corresponds to the clearance of EDTA from the body fluids by the kidney.

From the data obtained in the serial study of serum iron concentration following the intravenous administration of 3 Gm. of Ca EDTA to 4 subjects with hemochromatosis (Fig. 6), it appeared that in 3 of these subjects the drug had little, if any, effect on the serum iron level. It may be that the serum iron is so strongly bound by siderophilin that the drug is unable to chelate iron attached to this protein. The source of iron excreted in the urine may be iron that is chelated by the drug as it enters the blood stream from the gastrointestinal tract, from breakdown of red blood cells, or from storage in the tissues.

Why some subjects, especially those with hemochromatosis, excreted more iron than others, is not known. It is possible that iron entering the circulation from the afore-mentioned sources does so at a faster rate, or that it may be more loosely bound to protein and therefore more "available" for sequestration and excretion.

The maximal excretion of iron in the urine occurred in the subjects with hemochromatosis, and, although representing an eight- to tenfold increase over the basal output, was not in excess of 5 to 8 mg. in twenty-four hours. These results confirm and extend the observations reported by Wishinsky[3] in hemochromatosis. From a therapeutic point of view, a phlebotomy of 500 ml. of whole blood will remove approximately 250 mg. of iron and is thus a much more effective method of treatment. It is possible that the parenteral use of the drug or other iron chelating agents may be of value in secondary hemochromatosis where phlebotomy is contraindicated. This deserves further study.

While one obtains the impression that a rough separation can be made between patients with "normal" concentrations of serum iron and those with primary hemochromatosis, based upon the amount of iron chelated and excreted in the urine after a standard dose of Ca EDTA, it is obvious that there

was considerable overlapping in the two groups. From a diagnostic standpoint, the concentration of iron in the serum, and unsaturated iron-binding capacity may be a more reliable index of excess deposits of iron in tissues than the quantity appearing in the urine.

This was particularly apparent in Patients E. J. R., F. N., and F. D., all of whom exhibited a rather small response to Ca EDTA in spite of high levels of serum iron. The diminished response of E. J. R., who was clinically improved, may have been due to reduced tissue iron deposits subsequent to the removal of about 50 pints of blood during the preceding two and one-half years. In general, the correlation between concentrations of serum iron and ability to mobilize iron by chelation was unsatisfactory. The lowest serum iron value among the patients with hemochromatosis (G. W. B.) was 144 μg per cent, and yet this individual eliminated more iron in the urine after a 2 Gm. dose of Ca EDTA than any other subject.

In studying the metabolism of Ca EDTA, Foreman, Vier, and Magee[1] found that the material was poorly absorbed from the gastrointestinal tract of the rat and that 80 to 95 per cent of the dose could be accounted for in the feces, most of the animals absorbing between 2 to 4 per cent. In man, Foreman reported that a maximum of 5 per cent is absorbed.[10] The data accumulated in the present study support this assumption, since there was no increase in urinary iron even with doses of 10 Gm. daily given to patients who showed a substantial response to intravenous medication. Ca EDTA apparently does not prevent the absorption of food iron in the rat[9] or in man,[11] since experiments indicate that iron chelate is about as effective as ferrous sulfate in the cure of nutritional anemia. The two patients in the current series, A. M. and C. K., to whom Ca EDTA was administered orally during the course of an iron balance received only moderate quantities of iron from their food and retained small amounts of iron both before and after therapy. No effect of the material on fecal iron was noted.

A limiting factor in the use of this and other iron chelating drugs may be the rate of transport of iron from various sources to the serum, and not the potency of the chelating agent. Thus, intermittent administration of the drug may be more effective than daily doses; and a drug that is absorbed slowly and, therefore, is active for a longer period of time may be of greater therapeutic value.

Although no toxic effects were observed following the intravenous use of as much as 40 Gm. of the chelating agent during a five-day period, a word of caution seems advisable. Wishinsky and associates[3] reported a decrease in prothrombin activity. Dudley and co-workers[12] described fatal results in the treatment of 2 patients with hypercalcemia who received large doses of sodium EDTA. In both instances, uremia developed and was found to be associated with severe damage to the renal tubules, hemorrhagic manifestations, and enlargement of the reticuloendothelial cells. Estimations of prothrombin time in our patients, however, failed to demonstrate change in the activity of this clotting factor. Red and white blood cell counts remained

within the normal range in all subjects. Differential white blood cell counts were not affected and there was no decrease in the hemoglobin in any of the patients. One patient developed diarrhea while receiving the drug orally in a dosage of 9 Gm. per day, but his symptoms subsided promptly when medication was discontinued and no further deleterious effects were observed. Periodic examinations of the urine revealed no evidence of injury to the kidney.

SUMMARY

1. The urinary iron excretion is reported in 15 subjects including 7 with hemochromatosis, following the intravenous administration of Ca EDTA.

2. In the patients with hemochromatosis 4 Gm. of the drug was found to increase the excretion of iron in the urine from six- to tenfold. However, the maximum amount of iron recovered in a twenty-four-hour urine specimen did not exceed 8 mg.

3. In the subjects without hemochromatosis the maximum daily urinary iron did not exceed 3 mg.

4. Ca EDTA, when given orally in doses of 2 to 9 Gm. daily to 2 subjects with hemochromatosis and 1 with hypogonadism, did not increase the output of iron in the urine; nor was the iron balance of these two subjects affected by this treatment.

5. Red and white blood cell counts, differential counts, and determinations of hemoglobin remained within the normal range in all subjects. With the exception of 1 patient who developed diarrhea during oral administration of the drug, medication was well tolerated and no toxic effects were observed.

6. Intravenous Ca EDTA may be of some value in secondary hemochromatosis, but it is not a substitute for phlebotomy in primary hemochromatosis.

We wish to thank Dr. Leon Rosove for making available some of the patients used in this study, and also Mr. John D. Chilton for technical assistance in the analysis of urine and stool specimens.

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THE INFLUENCE OF HYPOCALCEMIA INDUCED BY SODIUM ETHYLENEDIAMINE TETRAACETATE ON THE TOXICITY OF POTASSIUM

An Experimental Study

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POTASSIUM intoxication is a common and important clinical problem. The diagnosis is made with increasing frequency due to a better understanding of predisposing factors, knowledge of helpful electrocardiographic patterns, and the greater availability of laboratory methods for potassium determination. Therapy, however, remains handicapped by an incomplete understanding of the factors which cause variable response to a given degree of hyperpotassemia and modify susceptibility to the toxic effects of potassium. Some of these modifying factors are the presence of myocardial disease,[1, 2] the blood pH,[3, 4] the rate of rise in potassium concentration,[5, 6] and the serum sodium concentration.[3, 5, 7] A factor which has not received the emphasis it deserves is the serum calcium level.

Knowledge of the critical importance of calcium dates to Ringer's demonstration[8] of the mutually antagonistic actions of calcium and potassium on myocardial contractility. Elevation of the serum calcium level manifests a protective action in experimental[9, 10] and clinical[11] potassium poisoning. The converse, the increased danger of an elevated serum potassium concentration when hypocalcemia is coexistent, might be inferred. However, there are few data obtained from intact mammals to support such an inference. The present work was designed to determine the effect of hypocalcemia on the tolerance to potassium intoxication in dogs.

METHODS

Mongrel dogs weighing 10 to 21 kg. (average 15 kg.) were anesthetized with morphine, 2 mg. per kilogram, subcutaneously, followed in 45 minutes by intravenous sodium pentobarbital, 16 mg. per kilogram. Additional small doses of the latter were given intermittently as needed to maintain light anesthesia. Artificial respiration with room air was provided by an endotracheal tube attached to a Starling pump.

From the Division of Cardiology, Philadelphia General Hospital and the Robinette Foundation of the University of Pennsylvania.

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Infusions were administered into a cannulated femoral vein. From an indwelling arterial needle, blood samples were obtained in heparinized syringes before and at frequent intervals during the infusions. Electrocardiograms were recorded at frequent intervals, utilizing a limb lead (Lead II or aV_F) and a unipolar chest lead (Lead V_2, V_3, or V_4). Care was taken to ensure that blood samples were obtained at the time of electrocardiographic changes and at death. Time of death was recorded as that moment when directly measured mean femoral artery blood pressure abruptly fell to 0 to 10 mm. Hg.

Plasma sodium and potassium were determined by internal standard flame photometry,[12] calcium by oxalate precipitation,[13] and whole blood pH with a model G Beckman pH meter.

Control Group.—After a preliminary anesthesia period of about one hour, nine animals were given infusions of 1.14 per cent potassium chloride in water at the rate of 0.06 meq. per kilogram per minute until death.

Hypocalcemia Group.—During earlier experiments not recorded here, an infusion technique was devised which allowed the production of a fairly stable level of hypocalcemia which, in itself, was not dangerous. For this purpose, the tetrasodium salt of ethylenediamine tetraacetic acid* (NaEDTAA) was used as a 2 per cent solution in 0.85 per cent saline. For 45 minutes, administration was at a rate of 2.5 mg. per kilogram per minute, after which the infusion was slowed to 1.25 mg. per kilogram per minute. After a 60-minute period of NaEDTAA infusion, potassium administration was begun as described for the control animals. The two groups were comparable in weight, duration of anesthesia and preinfusion levels of sodium, calcium, potassium, and pH.

RESULTS

The effect upon plasma calcium level of NaEDTAA infusion is illustrated in Fig. 1. Preinfusion calcium values of about 10 mg. per cent fell to from 4 to 5 mg. per cent by the end of 45 minutes. With slowing of the infusion at that time, the calcium concentration remained about half normal until termination of the experiment two hours later.

The toxic effects of potassium were compared in the experimental groups on two bases: (1) the potassium level at the time QRS duration increased by 0.02 seconds; and (2) the potassium level at the time of death.

In the 9 control animals, QRS widening was observed at potassium levels of 9.1 to 13.2 meq. per liter with a mean of 10.4 meq. per liter (S.D. 1.4). In the 9 animals with hypocalcemia, QRS widening was observed at potassium levels of 7.5 to 11.7 meq. per liter with a mean of 9.7 meq. per liter (S.D. 1.5) (Fig. 2). This difference in means appears to be of interest but is not statistically significant (probability of t equals 0.3). However, at potassium levels below 9.0 meq. per liter, QRS widening was seen in four of the nine hypocalcemic animals but in none of the control group.

The lethal level of potassium in the control group ranged from 11.6 to 15.4 meq. per liter with a mean of 13.2 meq. per liter (S.D. 1.2). In contrast, the hypocalcemic animals died when the potassium concentration rose to a level of from 10.0 to 13.4 meq. per liter with a mean of 11.4 meq. per liter (S.D. 1.0) (Fig. 3). It is worth noting that death occurred at serum potassium levels below 12.1 meq. per liter in 8 of the 9 hypocalcemic animals, but was noted in only 2 of the 9 control animals. The difference in mean potassium levels at the time of death (1.8 meq. per liter) in the two groups is statistically significant at the 1 per cent level.

*NaEDTAA was kindly supplied by Riker Laboratories, Los Angeles, Calif.

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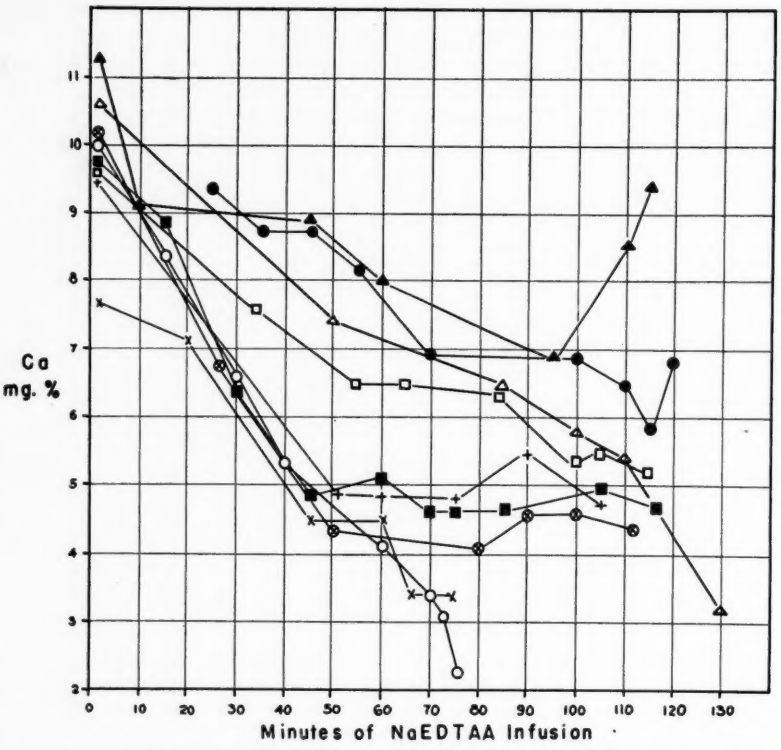

Fig. 1.—The effect of NaEDTAA infusion on the plasma calcium concentration. Nine dogs were given NaEDTAA intravenously at the rate of 2.5 mg. per kilogram per minute for 45 minutes, after which the rate was cut in half.

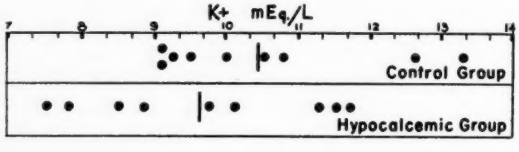

Fig. 2.—Plasma potassium concentration at the time of initial QRS widening. The mean value at the time of QRS widening is indicated by the vertical bar.

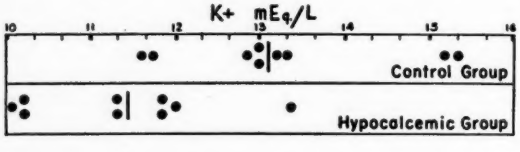

Fig. 3.—Plasma potassium concentration at the time of death. The mean value at the time of death is indicated by the vertical bar.

DISCUSSION

Mechanism of Action of NaEDTAA.—The biologic interest of the salts of EDTAA is related to their affinity for certain divalent and trivalent cations. The resultant sparsely ionized but water-soluble molecular complexes are termed "chelates." "Sequestration" is used to describe the functional removal of

metallic ions from solution by chelation. The pH of the solution is one of the variables which affect calcium sequestration by NaEDTAA and the stability of the resulting chelate. Throughout the range of blood pH values, stability of the calcium chelate is great[14]; the sequestered calcium is physiologically and chemically inactive and does not react with oxalate in the determination of plasma calcium.[15]

The extent of calcium sequestration is also influenced by competition of any other cation for which EDTAA has affinity. In plasma, however, the affinity of NaEDTAA for calcium is so much greater than for magnesium, copper, and zinc that these agents of uncertain physiologic significance are not chelated until calcium is nearly exhausted from solution.[15, 16]

Production of Hypocalcemia.—Previous experimental attempts to evaluate the effect of hypocalcemia on potassium toxicity have been hampered by inadequate methods of producing hypocalcemia of moderate degree.[17-19] Merklen and associates[17] demonstrated that oxalate injection accentuated the electrocardiographic signs of potassium poisoning in animals, but the influence of lowered serum calcium on lethal levels of potassium was not evaluated. In addition, the uncertain effects of products of oxalate metabolism, the brevity of the resulting hypocalcemia, and the marked change in heart rate cause difficulty in evaluating these results.

The use of NaEDTAA presents several advantages in a study of the effects of hypocalcemia. The toxicity of this agent in the doses used is limited to the effect of the hypocalcemia it produces,[16, 20, 21] and the hypocalcemia resulting from its infusion is fairly well maintained. Thus, in the hypocalcemic animals described above, depression of the calcium level had been present for one hour prior to death from hyperpotassemia. Finally, determination of the precipitable calcium after the use of NaEDTAA[14-16] does not present the laboratory difficulties inherent in determining plasma calcium after the use of oxalate or citrate.

Effects of Hyperpotassemia in the Presence of Hypocalcemia.—The properties of NaEDTAA make it possible to evaluate the effect of moderate hypocalcemia on potassium tolerance of dogs. As objective indices of potassium toxicity we selected (1) intraventricular conduction disturbance and (2) serum potassium concentration at the time of death. With regard to the first, our results are suggestive but not conclusive. As regards the second index, there is definite evidence that hypocalcemia in dogs is associated with a lower lethal level of plasma potassium. This finding appears to have the following clinical application: When a low serum calcium level complicates hyperpotassemia, therapy with calcium is important not only to repair a deficit of this electrolyte, but also to help counteract the toxic effects of potassium.

SUMMARY

1. A technique is described for the production in dogs of moderate, sustained hypocalcemia by the infusion of sodium ethylenediamine tetraacetate.

2. The effect of potassium chloride infusion in normal and hypocalcemic dogs was evaluated with respect to the plasma potassium concentration at the time of initial QRS widening and at the time of death.

3. There is suggestive evidence that hypocalcemia causes increased susceptibility to the toxic action of potassium as indicated by its effect on the intraventricular conduction time.

4. There is definite evidence that hypocalcemia causes increased susceptibility to the toxic action of potassium as indicated by the level of plasma potassium concentration necessary to cause death.

5. These observations would appear to have a clinical application in the management of potassium intoxication.

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ISONIAZID IN VITAMIN B_6-DEFICIENT RATS

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RECENT in vitro and in vivo studies[1-7] have indicated a possible antimetabolic relationship between isoniazid and pyridoxine. Some of the principal side effects of isoniazid have been associated with the central nervous system[5, 8, 9] and have resembled vitamin B_6 deficiency.[10, 11] Biehl and Vilter[5, 12] demonstrated peripheral neuritis in approximately 40 per cent of patients receiving 20 mg. per kilogram of body weight per day of isoniazid in the treatment of tuberculosis. They were able to prevent the neuritis by the addition of pyridoxine to the high dose of isoniazid.

Clinically, the effect of pyridoxine on the antituberculosis activity of isoniazid must be considered. Investigators[3] at this laboratory were able to reverse isoniazid inhibition with pyridoxine and its derivatives in some microorganisms, but were unable to do so in several strains of the Mycobacterium. Biehl and Vilter[5] and Ungar and associates[7] not only confirmed the in vitro work with Mycobacterium, but also showed that pyridoxine does not interfere with the antituberculosis activity of isoniazid in vivo. Pope[6] reported that pyridoxal hydrochloride could initiate growth of isoniazid inhibited H37Rv strain of *Mycobacterium tuberculosis* and completely neutralize isoniazid inhibition of endogenous respiration.

This study was undertaken to determine what relationship, if any, exists between pyridoxine and isoniazid in the rat. The effect on body and thymic weights, the occurrence of acrodynia and convulsions, and the excretion of xanthurenic acid before and after tryptophane were the characteristics of vitamin B_6 deficiency used to compare isoniazid to the known antimetabolite, desoxypyridoxine.

The data to be reported support the clinical observation of Biehl and Vilter[5, 12] in man and those reported by Rosen[13, 14] in vitamin B_6-deficient rats.

METHODS AND PROCEDURES

Eighty 6-week-old male Sprague-Dawley rats, with an average weight of 130 grams, were divided into 4 groups of 20 animals each. They were housed individually in wire metabolism cages. All animals received the basal vitamin B_6-deficient diet as described by Beaton and associates.[15] In addition the rats were given daily intraperitoneal injections as follows:

Group I	50 μg pyridoxine
Group II	100 μg desoxypyridoxine
Group III	50 mg./kg. isoniazid
Group IV	120 mg./kg. isoniazid plus 50 μg pyridoxine

Work done under the auspices of the AEC.
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*From the Los Alamos Scientific Laboratory of the University of California, Los Alamos, New Mexico.

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TABLE I. SUMMARY OF METABOLIC EFFECTS OF ISONIAZID IN VITAMIN B_6-DEFICIENT RATS

EFFECTS	GROUPS AND DIETARY TREATMENT*				
	I 50 μg PYRIDOXINE	II 100 μg DESOXY- PYRIDOXINE	III 50 MG. ISONIAZID /KILOGRAM OF BODY WEIGHT	IV 120 MG. ISONIAZID /KILOGRAM OF BODY WEIGHT PLUS 50 μG PYRIDOXINE	V DEFICIENT DIET ONLY
General Appearance	Normal	Poor	Very poor	Normal†	Fair
Average weight gain per rat (grams)‡					
Pair-fed	+16.3	+2.9 (p = .02-.05)	−7.0 (p = .01)	+8.0 (p = .05–.1)	--
ad libitum	+55.0	+5.5 (p = .01)	−14.9 (p = .01)	+30.5 (p = .01)	+17.0 (p = .01)
Average thymus weight per rat at 3 weeks (% of body weight)‡	0.35	0.13 (p = .01)	0.13 (p = .02)	0.31 (p = .01)	0.24 (p = .4)
Xanthurenic acid excretion (mg./24 hr.)					
Before tryptophane	0.20	0.26	0.27	0.33	0.21
After tryptophane	0.77	12.10	5.70	0.86	0.60
Convulsions	None	None	80%, early, severe	30-35%, late	None
Acrodynia	None	Severe	None	None	Mild

*All groups received the basal vitamin B_6-deficient diet supplemented daily as stated in the above column headings.

†Very few animals in this group had a poor appearance.

‡p-Values obtained by testing significance of difference between means when each group was compared with Group I (basal diet plus 50 μg pyridoxine).

Six additional animals were maintained throughout the experiment on ad libitum intake of the basal vitamin B_6-deficient diet alone (Group V).

Twelve rats from Groups I, III, and IV above were pair-fed according to the amount of food consumed the previous day by 12 corresponding animals in Group II (basal diet plus desoxypyridoxine). The remaining animals in each group were allowed ad libitum food intake. The pair-feeding and injections were continued without variation for 21 days. All animals were weighed twice each week and weight-variations between groups were analyzed for statistical significance.

All animals were observed daily for signs of vitamin B_6 deficiency and other metabolic disturbances. The specific observations were general appearance, weight loss, irritability, occurrence of acrodynia, onset of convulsive seizures, atrophy of the thymus, urinary excretion of xanthurenic acid before and after administration of tryptophane and reversal of isoniazid effects by pyridoxine. More specific descriptions of the methods used for the latter observations are given below.

Thymic Atrophy.—On the twenty-first day of the experiment, 4 pair-fed and two ad libitum-fed animals from each of the four experimental groups were sacrificed. The thymuses were dissected out and weighed and their weights expressed as per cent of the body weight. Testes, kidneys, and spleens were also weighed and any organ weight-variations between the various groups were subjected to statistical analysis.

Xanthurenic Acid Determinations Before and After Tryptophane.—After the twenty-first day, the remaining animals were maintained on ad libitum intake of the basal vitamin B_6-deficient diet, and the daily injections of the drugs as specified in each group were continued. Individual twenty-four-hour urine specimens were collected from 10 animals in each group for 9 days. On the sixth, seventh, and eighth days of this period, each of the animals received 200 mg. DL-tryptophane orally by stomach tube.

Xanthurenic acid determinations were made on the filtered urine specimens following the method of Glazer and associates.[16] A standard curve was obtained, according to the method of Rosen and co-workers,[17] using a solution of pure xanthurenic acid.* A Coleman Spectrophotometer, Model 14, at 620 mμ was used for all readings.

Reversal of Isoniazid Effects With Pyridoxine.—After 30 days, 13 animals that had received 50 mg. isoniazid per kilogram of body weight daily (Group III) and 11 animals that had received 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily (Group IV) were given increasing amounts of pyridoxine in an attempt to reverse the effects of isoniazid. Six animals in Group III received 20 μg and 7 received 200 μg pyridoxine daily for 3 days, after which the doses were increased to 200 and 500 μg, respectively. Five of the animals in Group IV had their dose of pyridoxine increased from 50 to 200 μg and 6 animals had their dose increased to 500 μg for 3 consecutive days, after which the doses were further increased to 500 and 1,000 μg, respectively. These animals were observed for reversal or alleviation of the various signs of metabolic disturbance from the isoniazid treatment.

RESULTS

The observations of the various metabolic effects of isoniazid in vitamin B_6-deficient rats are summarized in Table I. These results show the effect of vitamin B_6 deficiency alone, vitamin B_6 deficiency plus isoniazid, and vitamin B_6 deficiency plus desoxypyridoxine administration on general appearance, average weight gain following pair-feeding and ad libitum food intake, average thymus weight loss, excretion of xanthurenic acid before and after tryptophane administration, onset of convulsions, and appearance of acrodynia. These

*Xanthurenic acid (m.p. 297°-299° C.) kindly supplied by Dr. Fred Rosen of the Ortho Research Foundation, Raritan, N. J.

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results show that the general appearance of the animals which received the basal vitamin B_6-deficient diet plus 50 μg pyridoxine and the basal diet plus 120 mg. isoniazid per kilogram of body weight and 50 μg pyridoxine was essentially normal, while the general appearance of those animals that received the basal diet plus 100 μg desoxypyridoxine, and the basal diet plus 50 mg. isoniazed per kilogram of body weight was very poor. The general appearance of a typical animal from each of the four experimental groups on the twenty-first day of the experiment is shown in Fig. 1.

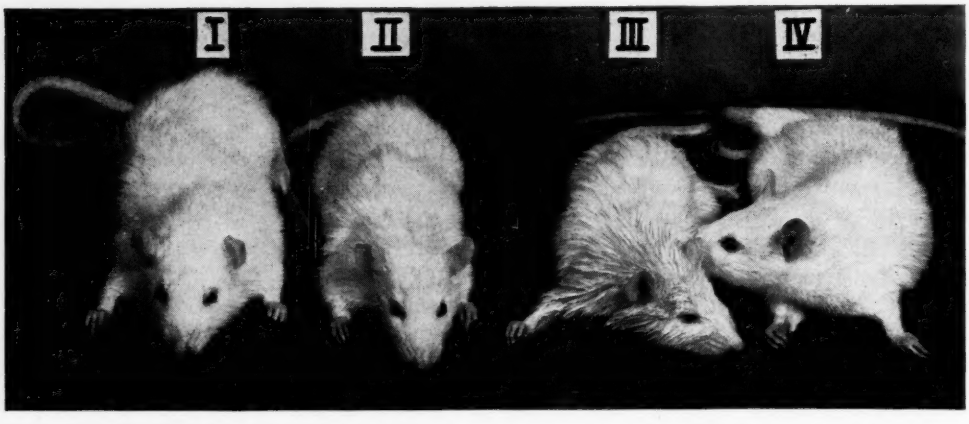

Fig. 1.—General appearance of animals 21 days from beginning of experiment. Group I. Basal diet plus 50 μg pyridoxine daily. Group II. Basal diet plus 100 μg desoxypyridoxine daily. Group III. Basal diet plus 50 mg. isoniazid per kilogram of body weight daily. Group IV. Basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily.

Only those animals that received the basal vitamin B_6-deficient diet plus 50 mg. isoniazid per kilogram of body weight daily showed a weight loss at the end of the experimental period of 21 days. Under conditions of pair feeding and ad libitum food intake this group showed an average weight change of –7.0 and –14.9 grams per rat, respectively, compared to an average of +16.3 and +55.0 grams, respectively, for the animals in the control group (basal diet plus 50 μg pyridoxine). The group that received 100 μg desoxypyridoxine daily in addition to the basal diet showed a very small weight gain, and animals that received 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine, gained approximately half the amount of the control group. The animals on ad libitum intake of the basal vitamin B_6-deficient diet alone showed an average weight gain which was about one-third that of the controls. The general trend of the weight gains among the various groups was essentially the same, whether the animals were pair-fed or received food on ad libitum intake.

After 21 days, on the specified regimens, the animals that received the basal diet plus desoxypyridoxine and the animals that received the basal diet plus 50 mg. isoniazid per kilogram of body weight showed essentially the same thymic weights. The average weight of the thymuses in each of these groups was 0.13 per cent of body weight. The control group, which received the basal diet

plus 50 μg of pyridoxine daily, and the group that received the basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine, had comparable thymic weights, constituting 0.35 and 0.31 per cent of body weight, respectively. The group on the basal diet alone had an average thymic weight of 0.24 per cent of body weight. There were no significant weight differences in the kidneys, testes, or spleens of the animals in the various groups.

There was no great change in the amount of xanthurenic acid excreted in the urine of the various groups before the administration of tryptophane. After the administration of tryptophane, urinary excretion of xanthurenic acid by the animals that received the basal diet alone, the basal diet plus 50 μg pyridoxine and the basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine was not greatly increased. However, xanthurenic acid excretion by the animals on the basal diet plus 100 μg desoxypyridoxine was increased fiftyfold and excretion by animals on the basal diet plus 50 mg. isoniazid per kilogram of body weight was increased twentyfold. The effect of the various regimens on the excretion of xanthurenic acid in the urine before and after the administration of tryptophane is more clearly illustrated by the normogram given in Fig. 2.

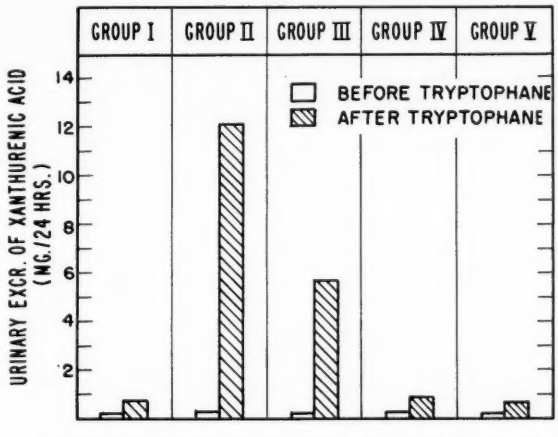

Fig. 2.—Effect of various regimens on the urinary excretion of xanthurenic acid before and after tryptophan. Group I. Basal diet plus 50 μg pyridoxine daily. Group II. Basal diet plus 100 μg desoxypridoxine daily. Group III. Basal diet plus 50 mg. isoniazid per kilogram of body weight daily. Group IV. Basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily. Group V. Basal pyridoxine—deficient diet only.

Convulsions occurred only in the groups of rats that received isoniazid. Within two weeks after the beginning of the experiment, animals that had received the basal vitamin B_6-deficient diet plus 50 mg. isoniazid per kilogram of body weight were hyperirritable, and on the sixteenth day one animal convulsed. From the sixteenth day on, a progressively increasing number of animals had convulsive seizures and, by the twenty-first day, 80 per cent of the animals in this group were so affected. The few animals that did not convulse showed signs of hyperirritability. The animals that received 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily showed much less

tendency toward early convulsions. A few, however, convulsed near the end of the third week, and 35 per cent of the animals had convulsive seizures by the end of the twenty-ninth day. Several of the convulsions occurred during the stress period of oral administration of tryptophane. Before the twenty-ninth day, one animal in each of the two afore-mentioned groups died respiratory-type deaths during the course of the convulsive seizures.

TABLE II. EFFECT OF PYRIDOXINE THERAPY ON CONVULSIONS OF ISONIAZID-TREATED VITAMIN B_6-DEFICIENT RATS

	NUMBER OF ANIMALS	TREATMENT	% OF ANIMALS CONVULSING
III (Basal diet plus 50 mg. isoniazid per kilogram of body weight daily for 30 days)	6	None	83
		20 μg pyridoxine daily for 3 days	50
		20 μg pyridoxine daily for 3 days, followed by 200 μg daily for 3 more days	0
	7	None	85
		200 μg pyridoxine daily for 3 days	0
		200 μg pyridoxine daily for 3 days, followed by 500 μg daily for 3 more days	0
IV (Basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily for 30 days)	5	None	20
		Pyridoxine increased 50 μg 3 days	50
		Pyridoxine increased 50 μg daily for 3 days, followed by 500 μg daily for 3 days	0
	6	None	50
		Pyridoxine increased 500 μg daily for 3 days	0
		Pyridoxine increased 500 μg daily for 3 days, followed by 1,000 μg daily for 3 more days	0

Data showing the effect of pyridoxine administration on the alleviation of convulsions of isoniazid-treated, vitamin B_6-deficient rats are summarized in Table II. These data show that convulsions, in the group that had received the deficient diet plus 50 mg. isoniazid per kilogram of body weight daily for 29 days, were completely alleviated by daily administration of 200 μg pyridoxine for 3 days. No further convulsions occurred in the animals that had received 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine for 29 days, after the dose of pyridoxine was increased to 500 μg for 3 days.

On the second day of treatment with pyridoxine, one animal in the group that had received the basal vitamin B_6-deficient diet plus 50 mg. isoniazid per kilogram of body weight died during a convulsion. This animal was receiving only 20 μg pyridoxine at the time and was extremely cachectic and emaciated.

Another animal in this same group died after the sixth day of pyridoxine injections. The animal did not die during a convulsion but was so debilitated that undoubtedly permanent irreversible damage had occurred. One animal in the group that had received 120 mg. isoniazid per kilogram of body weight plus 50 μg. of pyridoxine, convulsed before the pyridoxine was increased over the initial 50 μg daily. Two animals had mild convulsions in this group even after 500 μg pyridoxine had been given for 1 day. One of these animals died, the other survived and had no subsequent seizures. The severity of the degree of metabolic disturbance in the isoniazid-treated animals at the time pyridoxine therapy was started and its complete reversal is shown in Fig. 3. All surviving animals in the two groups that had received isoniazid gained weight rapidly

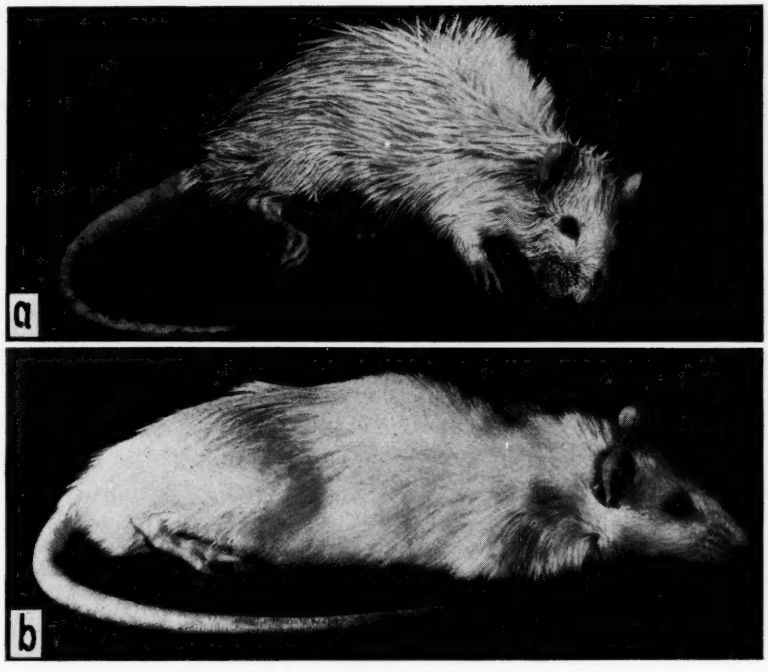

Fig. 3.—General appearance of isoniazid-treated rats before and after pyridoxine therapy. *a*, Basal diet plus 120 mg. isoniazid per kilogram of body weight plus 50 μg pyridoxine daily for 30 days. *b*, Same animal after therapy with 500 μg pyridoxine daily.

after the treatment with pyridoxine was started or increased. The urine of the animals in the group that had received 50 mg. isoniazid per kilogram of body weight was free from abnormal amounts of xanthurenic acid after the beginning of pyridoxine therapy. These animals were sacrificed when their body weights returned to normal, and the thymuses were weighed and found to be within normal limits.

Acrodynia was very severe in the group that had received the basal vitamin B_6-deficient diet plus desoxypyridoxine and mild in the group that had received the basal vitamin B_6-deficient diet only. No acrodynia was observed in any of the animals that received isoniazid, regardless of pyridoxine intake.

DISCUSSION

Objectively, the animals that received the basal vitamin B_6-deficient diet plus 50 mg. isoniazed per kilogram of body weight daily (Group III), were in the poorest general condition.

In the present study only 50 mg. isoniazid per kilogram of body weight daily was required to produce convulsions and a negative weight gain in rats that were on a vitamin B_6-deficient diet. Rubin and associates[18] found that four times the maximum accepted oral dose of isoniazid was necessary to decrease the normal rate of weight gain of rats on a normal diet and that doses of 320 to 640 mg. per kilogram daily were required to produce a net weight loss. At these doses some of the animals convulsed after a week or more. Comparison of the present results with those reported by Rubin and co-workers indicate that isoniazid is 6 to 12 times as effective at producing metabolic disturbances in vitamin B_6-deficient animals as it is in animals on a normal diet.

The animals that received the basal diet plus 120 mg. isoniazid per kilogram of body weight and 50 μg pyridoxine daily (Group IV) appeared in much better condition than those in Groups II and III and their general appearance approached that of the controls (Group I). Pyridoxine in daily doses of only 50 μg showed considerable protection against the metabolic effects of 120 mg. isoniazid per kilogram of body weight per day in rats that were otherwise deficient in vitamin B_6.

Vitamin B_6 deficiency is known to retard growth of lymphoid tissue[19, 20] and desoxypyridoxine is known to enhance the weight loss of the thymus of vitamin B_6-deficient animals. That isoniazid acts similarly to desoxypyridoxine in enhancing the weight loss of the thymus of vitamin B_6-deficient animals is shown by comparing the thymic weights of animals in Groups II (basal diet plus 100 μg desoxypyridoxine) and III (basal diet plus 50 mg. isoniazid per kilogram of body weight daily). Both isoniazid and desoxypyridoxine enhanced the retardation of growth of lymphoid tissue, and the average thymus weights of these two groups were significantly less than the average of the group that received the vitamin B_6-deficient diet alone (Group V).

A pronounced urinary excretion of xanthurenic acid after oral administration of tryptophane is associated with vitamin B_6 deficiency. The results show that a large dose of isoniazid does interfere with the normal metabolic pathways of tryptophane but not to the same extent as much smaller doses of desoxypyridoxine. That such large amounts of isoniazid are necessary to produce effects similar to desoxypyridoxine might be explained by postulating that isoniazid is not the active toxic principle but one of its metabolic products may be responsible for the antimetabolic action.

During these studies convulsions occurred only in those animals that received isoniazid in addition to the vitamin B_6-deficient diet. These convulsions, without exception, occurred 30 to 60 minutes after injection of the isoniazid. It was possible to prevent these convulsions and reverse the signs in nearly every case by giving a sufficient dose of pyridoxine intraperitoneally approximately one hour before the daily dose of isoniazid. In only two or three animals had

irreversible changes taken place during the entire experimental period. All the signs and symptoms of metabolic disturbance and isoniazid toxicity rapidly disappeared shortly after the animals were placed on sufficient doses of pyridoxine.

Acrodynia, which is a common sign of vitamin B_6 deficiency, was very pronounced in the animals receiving the basal diet plus desoxypyridoxine and was mild in the animals on the vitamin B_6-deficient diet alone. It never occurred in any of the animals receiving isoniazid. Convulsions, however, which have been described as occurring after very prolonged periods of vitamin B_6 deficiency, occurred early in the animals receiving isoniazid and never occurred in the vitamin B_6-deficient animals. These observations agree with those reported by Rosen[13, 14] who indicated that not all of the effects of isoniazid are similar to vitamin B_6 deficiency.

It would be difficult from these experiments alone to conclude that the sites of action of desoxypyridoxine and isoniazid were the same, but it has been shown that some of the signs associated with vitamin B_6 deficiency resemble the toxic signs of isoniazid. Also, isoniazid toxicitiy is enhanced in vitamin B_6-deficient animals.

Biehl and Vilter[5] have demonstrated an increased urinary excretion of pyridoxine in patients on isoniazid with irregular appearance of abnormal amounts of xanthurenic acid. They felt that their data did not support the thesis of isoniazid as a metabolic antagonist. Whether isoniazid is considered an antimetabolite may be a matter of definition. Woolley[21] has pointed out that all biological effects of a compound need not be related to its antimetabolic properties even though some of them may be. He also emphasized that an antimetabolite need not harm all species of higher animals or all microorganisms. The apparent relationship of isoniazid to pyridoxine need not explain all the actions of the drug, and indeed, may not exist in some systems of organisms.

If one considers an antimetabolite as an agent of similar structure which has the ability to produce a deficiency of a specific metabolite, whether this objective is obtained through exclusion of the metabolite from participating in enzymatic reaction or by its chemical alteration, or by some other means, then, isoniazid must be considered an antimetabolite. This hypothesis is substantiated by experiments showing that bacterial inhibition by isoniazid is reversed competitively by pyridoxine and its derivatives[3] and by this study wherein toxic symptoms of isoniazid similar to vitamin B_6 deficiency are reversed by pyridoxine in the rat.

In any event the results obtained in the present study further emphasize the possible clinical significance of vitamin B_6 deficiency or depletion in isoniazid therapy.[5, 12]

SUMMARY

Rats fed a diet deficient in vitamin B_6 and injected daily with the maximum acceptable oral dose of isoniazid showed a greater retardation of growth than rats receiving the same diet plus the known antimetabolite, desoxypyridoxine.

The thymus gland had atrophied to the same proportion of body weight in each of these two groups. In animals receiving either desoxypyridoxine or isoniazid, urinary xanthurenic acid excretion was 20 to 50 times greater after oral tryptophane than in animals receiving the basal diet plus pyridoxine or the basal diet alone.

Acrodynia was very severe in the animals receiving desoxypyridoxine but never occurred in any of the animals receiving isoniazid. Convulsions occurred only in the animals receiving isoniazid and were more pronounced in the animals receiving the maximum acceptable oral dose of isoniazid with no pyridoxine than in the animals receiving over twice as much isoniazid plus 50 μg pyridoxine daily. Convulsions never occurred in the animals on the vitamin B_6-deficient diet only or in those on the basal diet plus 100 μg desoxypyridoxine.

The animals on the deficient basal diet plus 120 mg. isoniazid per kilogram of body weight daily were protected against severe retardation in growth, thymus atrophy, and excretion of xanthurenic acid in the urine by 50 μg pyridoxine daily. This amount of pyridoxine partially protected the animals from convulsions.

Some, but not all, of the signs and symptoms of metabolic disturbance associated with vitamin B_6 deficiency resemble the signs of isoniazid toxicity. Isoniazid appeared to be 6 to 12 times more effective in producing metabolic disturbances in vitamin B_6-deficient animals than in animals on a normal diet.

It was possible to prevent convulsions in isoniazid-treated animals if a sufficient dose of pyridoxine was given 30 to 60 minutes before the isoniazid and all signs of metabolic disturbance and isoniazid toxicity rapidly disappeared shortly after the animals were placed on sufficient doses of pyridoxine.

We are grateful to Dr. C. C. Lushbaugh and Miss Dorothy Hale for the autopsies of the animals and the organ weight measurements.

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THE EFFECT OF AMINO ACID–GLUCOSE INFUSIONS UPON THE SERUM AMINO ACID AND BLOOD SUGAR CONCENTRATIONS IN VIRAL HEPATITIS

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INTRODUCTION

FOLLOWING a standard breakfast containing approximately 20 Gm. of protein there is a considerably greater and more prolonged rise in the serum amino acid concentration among normal individuals than there is among patients with hepatitis.[1] Von Falkenhausen[2] in 1924 originally observed a similar disparity following the oral administration of amino acids to a few jaundiced patients, and he concluded that the absence of bile from the intestine was responsible for relatively poor absorption of the amino acids. Von Falkenhausen's hypothesis does not seem tenable because we know of no presently recognized reason for supposing that a patient with hepatitis who is hungry and eating well should fail to absorb amino acids. In fact, the stools of jaundiced individuals, whether they be hungry or nauseated, do not ordinarily contain abnormal amounts of nitrogen, unless there is obstruction of the pancreas or some other collateral cause.

A direct approach to the problem was attempted by following the blood amino acid concentration after the infusion of amino acids.

MATERIAL AND METHODS

The subjects of the experiment were 10 patients with viral hepatitis and 13 controls. The diagnosis of hepatitis was established by the history, the laboratory data, and the course of the illness. All of these patients with hepatitis recovered, and only one of them was ever critically ill. The serum bilirubin when the tests were performed was between 1.9 and 34.0 mg. per cent. The control subjects were 4 normal ambulatory individuals and 9 hospitalized patients who had either no discernible organic disease or an illness such as healing peptic ulcer which was likely not to impair liver function.

Each subject was infused with 500 c.c. of distilled water containing 5 per cent amino acids derived from proteolytic digestion of casein and 5 per cent glucose.* The solution was infused in a 35- to 45-minute period. This degree of rapidity was accompanied by a mild sensation of "prickling" or "flushing," particularly around the head and neck, in about one-half of the cases. The infusion provided about the same amount of protein as had been given orally in a previous experiment,[1] and the glucose was included because the test meal in the previous experiment had contained carbohydrate.

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*Five per cent "Amigen" with 5 per cent glucose, generously supplied by Mead Johnson & Co.

Just before the start of the infusion and at 1, 2, 3, and 4 hours after the start of the infusion, a blood specimen was obtained for amino acid determination, and in 8 of the controls and 5 of the patients with hepatitis similarly timed blood specimens were obtained for blood sugar analysis.

Amino acid nitrogen was determined by the copper method of Albanese and Irby, as previously described,[1] and blood sugar was measured by the Lauber and Mattice modification of the Folin-Wu method.[3] All chemical determinations were performed in duplicate.

RESULTS

The average fasting amino acid level was almost the same in the two groups: 5.9 mg. per cent among the controls, and 6.0 mg. per cent among the patients with hepatitis. The average changes in the amino acid levels following the infusions are depicted in Fig. 1. It would appear from the graph that at all points after the fasting level the hepatitis curve is lower. Statistical comparison of various characteristics of the two curves, however, is shown in Table I, and it can be seen that there is no significant difference between the control and hepatitis groups with respect either to the rise above the fasting level or to the fall from peak to nadir. There is, on the other hand, a significant difference between the two groups in considering these 4 features: (1) ratio of the rise to the fall; (2) difference between the fasting level and the nadir in mg. per cent. (3) difference between the fasting level and the nadir in per cent of the fasting level; and (4) average 4-hour difference from the fasting level.

TABLE I. STATISTICAL COMPARISON OF DIFFERENCES BETWEEN AMINO ACID CURVES IN CONTROL SUBJECTS AND PATIENTS WITH HEPATITIS FOLLOWING INFUSION OF 5 PER CENT GLUCOSE AND 5 PER CENT AMINO ACIDS

CHARACTERISTIC OF THE CURVE	CONTROL MEAN	HEPATITIS MEAN	STANDARD DEVIATION OF DIFFERENCE BETWEEN MEANS	"T"	"P"
Rise above fasting level in mg. %	3.4	2.4	1.6	1.6	>0.10
Rise above fasting level in % of fasting level	58.3	40.9	28.3	1.5	>0.10
Fall in mg. % below peak	3.7	3.3	1.4	1.1	>0.25
Fall in % of rise	112.6	142.0	29.4	2.3	<0.05
Lowest value in mg. % below fasting level	−0.3	−0.9	0.8	1.8	Approx. 0.05
Lowest value in % change from fasting level	−3.8	−15.0	11.6	2.3	<0.05
Average % change during 4 hours	20.3	3.8	14.3	2.6	<0.025

The curves were also compared with curves in similar patients and controls previously given 20-Gm. protein test meals instead of amino acid infusion.[1] These data, summarized in Table II, reveal no significant difference between the postprandial and postinfusion curves among the patients with hepatitis except for the obviously higher peak following the infusions. Similarly, among the controls there is no great difference between the two groups apart from the higher peak values after infusion. It thus appears that those features of the postinfusion curves that most clearly divide hepatitis from the controls are not greatly altered by the route through which the amino acids are absorbed.

TABLE II. STATISTICAL COMPARISON OF DIFFERENCES BETWEEN AMINO ACID CURVES FOLLOWING MEAL AND FOLLOWING INFUSION

	HEPATITIS PATIENTS					CONTROLS				
CHARACTERIC OF CURVE	"ORAL" MEAN	"I.V."	S.D.	"T"	"P"	"ORAL" MEAN	"I.V."	S.D.	"T"	"P"
Highest value in mg. % above fasting level	0.5	2.4	0.5	9.1	<0.0005	1.1	3.4	0.6	3.7	<0.0005
Highest value in % change from fasting level	2.1	40.9	10.7	9.0	<0.0005	17.6	58.3	25.8	3.9	<0.0005
Lowest value in mg. % below fasting level	−0.8	−0.9	0.6	0.9	>0.25	0.1	−0.3	0.8	1.2	>0.10
Lowest value in % change from fasting level	−11.3	−15.0	8.5	1.1	>0.25	2.5	3.8	10.8	1.4	$>>0.10$
Average % change during 4 hours	−4.0	+3.8	6.2	1.7	>0.10	10.0	20.3	13.3	1.9	Approx. 0.05

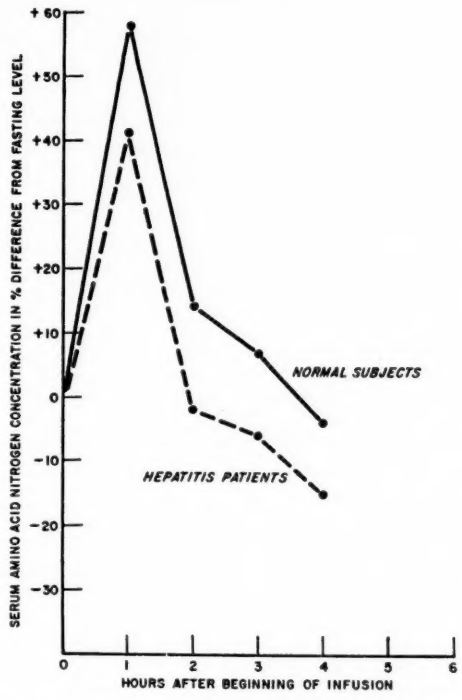

Fig. 1.—Serum amino acid concentration following infusion of 500 c.c. 5% amino acids and 5% glucose in 13 normal subjects and in 10 patients with hepatitis.

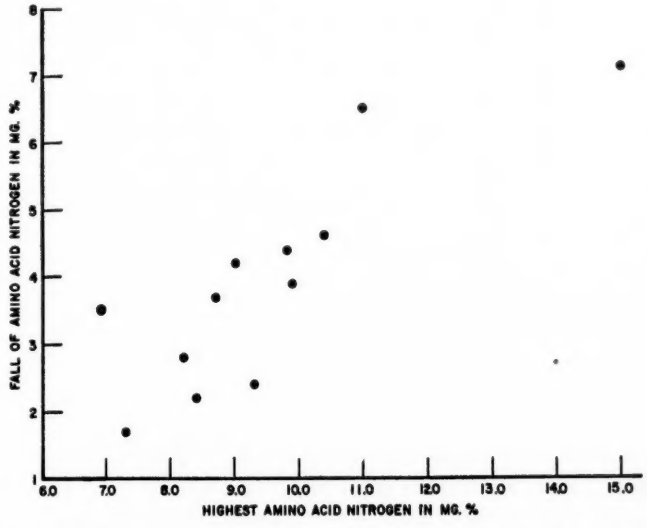

Fig. 2.—Relationship between the highest amino acid concentration and the fall in amino acid nitrogen following infusion of amino acids in 13 normal individuals.

As in the previous experiment,[1] calculations were made in each group for the correlation between the fasting level and the fall below the fasting level. This correlation coefficient in the hepatitis group was highly significant, while among the controls there was no such correlation (Table III).

TABLE III. CORRELATION COEFFICIENT ("R") FOR AMINO ACID DATA

HEPATITIS PATIENTS	"R"	"P"
Fasting level and fall below fasting level	0.8257	<0.01
Highest level and fall from highest level	0.7390	approx. 0.01
Rise to highest level and fall from highest level	0.3270	>0.10
CONTROLS		
Fasting level and fall below fasting level	0.0495	>0.10
Highest level and fall from highest level	0.8337	<0.01
Rise to highest level and fall from highest level	0.9110	<0.01
DATA OF J. MURRAY LUCK AND ASSOCIATES		
Fasting level in normal human subjects and maximum fall following injection of insulin	0.6985	<0.05
Fasting level in rabbits and fall following injection of epinephrine	0.001	$>>0.10$

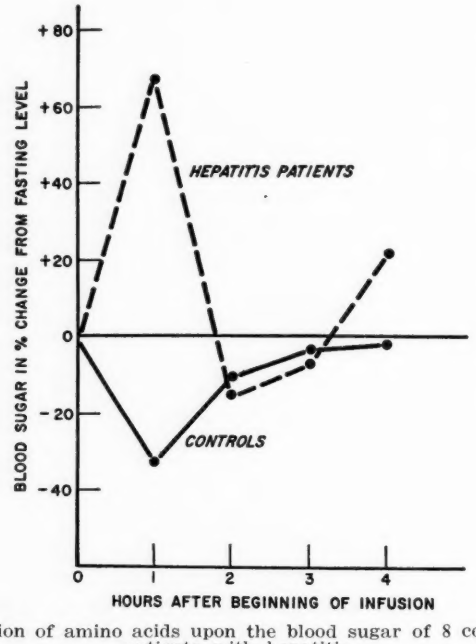

Fig. 3.—Effect of infusion of amino acids upon the blood sugar of 8 control subjects and of 5 patients with hepatitis.

It is also apparent (Table III) that there is a high correlation between the peak amino acid level and the fall of the amino acid level in both groups. Among the controls, but not among the patients with hepatitis, an even higher correlation was found between the rise and the fall. Fig. 2 illustrates the obvious correlation between the peak and the fall among the controls.

Fig. 3 is a graph of the average changes in the blood sugar concentrations. At the first hour after the start of the infusions the blood sugar among the hepatitis patients is high, and among the controls it is low. Thereafter, the

differences between the two curves are much less marked, but the hepatitis 4-hour average and average concentration at the fourth hour are significantly higher than their counterparts (Table IV).

TABLE IV. STATISTICAL COMPARISON OF BLOOD SUGAR CURVES

	HEPATITIS MEAN	CONTROL MEAN	S.D. OF DIFF. BETWEEN MEANS	"T"	"P"
Average 4-hour level in % change from fasting level	+13.8	−12.6	12.6	3.4	<0.001
4th hour average % change from fasting level	+22.5	−3.0	16.0	2.8	<0.025
Fasting blood sugar in mg. %	49.0	63.0	7.0	3.1	<0.001

DISCUSSION

Under the conditions of this experiment the postinfusion amino acid curves in hepatitis were not as strikingly different from the normal response as were comparable curves following the ingestion of a 20-Gm. protein meal.[1] That differences in amino acid metabolism might be masked by the infusion of as much as 25 Gm. of protein in 45 minutes should have been expected. In a way, this procedure is like trying to measure the influence of emotion on heart rate while the subjects are running and already have tachycardia. The very high peak amino acid concentration induced by the infusion would never occur naturally, apart from such extreme and dire diseases as acute yellow atrophy of the liver.[4]

Nevertheless, there was a clearly demonstrated difference between the control and hepatitis amino acid curves. The hepatitis curves fell to a lower level, and, in this respect, the curves here reported certainly mimic the postprandial amino acid curves previously described. Indeed, the amino acid blood levels at the fourth hour are not significantly different in the two experiments (Table II). It seems, therefore, that the tendency of the postprandial amino acid level to fall greatly in hepatitis is not due to poor digestion of the protein consumed nor to poor absorption of the products of digestion.

Once again there is clearly demonstrated a tendency for the hepatitis patient as well as the control to reduce the amino acid blood concentration in direct proportion to the height of the blood level when the fall begins (Fig. 2, Table III). That is to say, the higher the blood amino acid concentration, the greater the fall in this concentration. Since it is well known that insulin acts in this same manner upon glucose,[5] it seems reasonable to inquire whether insulin may similarly affect the blood amino acids. Analysis of the original work of Luck and his co-workers,[6] demonstrating that insulin depresses the serum amino acid concentration, supports this concept. Luck measured the serum amino acid concentration in 10 normal individuals in the fasting state, then injected insulin, and followed the serum amino acid levels at hour intervals for 5 hours. In Fig. 4 these fasting levels are plotted on the x-axis, and the lowest concentration observed in 5 hours plotted on the y-axis. The nearly linear relationship is strikingly revealed, and the correlation coefficient relating these data is 0.6985,

a finding expected by chance less often than 1 time in 20 (Table III). Furthermore, the regression coefficient for the effect of the fasting level upon the post-insulin fall was 0.5424, which differs insignificantly from the regression coefficient (0.6326) for the influence of the peak upon the fall. By way of contrast, there was no correlation at all between the fasting amino acid concentration and the fall in serum amino acid concentration following Luck's[7] administration of epinephrine to rabbits (Table III).

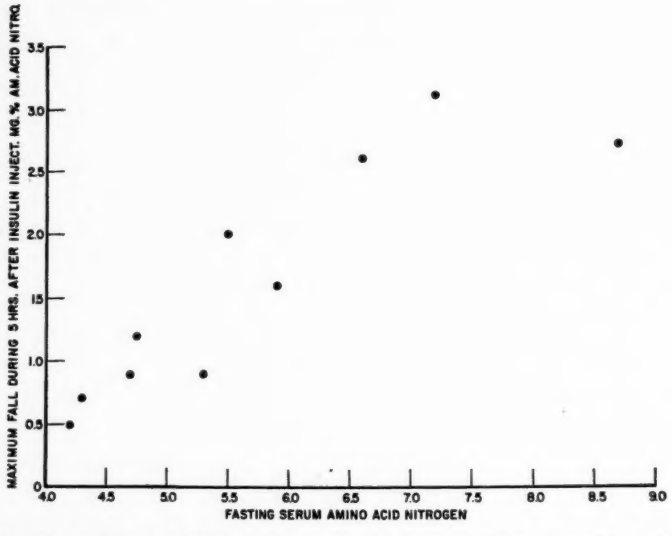

Fig. 4.—Relationship between fasting amino acid nitrogen and fall in amino acid nitrogen following insulin injection. (Chart prepared from data of Daniels and Luck.[6])

These observations by no means constitute proof that insulin is the factor responsible for the postabsorptive fall in the serum amino acid concentration, but the data are not incompatible with such an hypothesis.

The difference between the control and hepatitis blood sugar concentrations is probably a reflection of the damaged liver's difficulty in storing glycogen.[8] There is, however, a remarkably low blood sugar concentration at the end of 1 hour among the controls (Fig. 3). In 3 individuals the blood sugar concentration at 1 hour was below 25 mg. per cent, and there were obvious clinical signs of hypoglycemia. This unexpected phenomenon may be explained by the tendency of rapidly infused amino acids to depress the blood sugar concentration.[9]

SUMMARY

1. Changes in the serum amino acid concentration were followed in 13 normal individuals and in 10 patients with viral hepatitis following infusion of 500 c.c. of distilled water containing 5 per cent amino acids and 5 per cent glucose.

2. The changes were similar to those previously observed following a 20-Gm. protein meal: In hepatitis the amino acid concentration, after an initial rise, falls farther than it does in the controls.

3. The blood sugar concentration appeared to fall remarkably after the infusions in the controls, but in the patients with hepatitis there was a greater tendency for the blood sugar to rise.

4. Correlation and regression coefficients found between certain sets of these data and in data accumulated by Daniels and Luck in 1931 suggest that the postinfusion or postprandial fall in the serum amino acid concentrations is caused by insulin, and that the magnitude of serum amino acid depression caused by insulin is proportional to the serum amino acid concentration.

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PEPSINOGEN IN THE BLOOD

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THE measurement of enzymes in the blood for clinical purposes is fairly recent; measuring such enzymes as amylase and lipase and the phosphatases has by now become standard laboratory procedure.

Among other enzymes which have been studied in relation to disease, pepsinogen in the blood, though first described over forty years ago,[11] has only recently drawn clinical attention.[7, 8] While some of the disturbances in disease, notably in duodenal ulceration and in pernicious anemia, have been described,[3, 8] much has still to be learned concerning its relation to gastric function and to pathologic physiology in gastroduodenal disease. If the relation to gastric function can be clearly established, both in normal and in pathologic circumstances, the measurement of plasma pepsinogen might be a useful tool in the long-term study of peptic ulceration and its treatment.

Results presented here concern the identity of the enzyme in the blood, its distribution in the blood plasma and cells, its relation to gastric pepsin secretion, and its disturbances in a small number of gastric and duodenal ulcer patients.

METHODS

The Anson[1] hemoglobin method for peptic assay was modified to measure pepsinogen in the blood and in similar fluids. Two per cent bovine hemoglobin enzyme substrate (Wilson) in 0.05 N HCl (pH 1.7) was freshly made up for each day's assays from a 10 per cent aqueous stock solution. Five milliliters of this substrate (= 0.1 Gm. Hb.) was incubated at 37° C. with 1 ml. of plasma or serum for two hours and the reaction then stopped with 5 ml. 0.6 M trichloracetic acid. A parallel control blank was prepared for each assay by precipitating a plasma-substrate mixture at the outset. The precipitated mixture should be warmed at 37° C. for 10 to 20 minutes to facilitate filtration (Whatman No. 41).

To determine the increment of tyrosine-like products of digestion, 2 ml. of filtrate was made alkaline with 4 ml. of 0.5 N NaOH, 1.2 ml. of freshly diluted Folin-Ciocalteau reagent was added, and the mixture was immediately shaken, and read in a spectrophotometer 3 to 20 minutes later at 560 mμ, with the blank zeroed in the machine. One arbitrary peptic unit (P.U.) is the spectrophotometric equivalent of 1 x 10^{-4} meq. of l-tyrosine (0.0181 mg.) released in a standard hemoglobin assay in ten minutes, and designated (P.U.) $\frac{\text{Hb.}}{10''}$, abbreviated to P.U. The ten-minute period was derived by simple linear extrapolation from the two-hour incubation period used, supported by the finding that the products of digestion are linear from 2 to 24 hours[7] and confirmed by myself for the period 10 to 120 minutes. Results are expressed as P.U. per 100 ml. (P.U. per cent.)

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Incubation Time.—Two-hour and 24-hour incubation periods were compared in a number of instances; the standard deviation of the different samples on the same subject in all studies was ±22.4 per cent; that by the 24-hour incubation ±9.2 per cent. However, plasma containing over 40 P.U. per 100 ml. produces such a large increment of chromogenic substances in the 24-hour incubation period as to necessitate further dilution of the filtrate and thus introduces a further potential error. For general clinical use, therefore, the two-hour method has the advantage of time and avoidance of overdigestion of substrate, which outweighs the somewhat greater inaccuracy than found in the 24-hour incubation. Where very low values are anticipated, the 24-hour incubation would be advisable.

RESULTS

Identity of the Enzyme.—Human blood exhibits proteolysis in the whole pH range from 1 to 6 (Table I). Proteolysis below pH 3.5 is probably peptic[7] and between 3.5 and 6.0 probably catheptic.[6, 7]

TABLE I. HEMOGLOBIN DIGESTION BY POOLED PLASMA AND WHOLE BLOOD FROM THE SAME SOURCES AT pH 1.0 TO 6.0

pH	1.0	2.0	3.0	4.0	5.0	6.0
Plasma "P.U."%	23	48	54	33	16	48
Whole Blood "P.U."%	59	40	68	57	60	40

Mirsky and associates[7] claim, however, that the digestion of hemoglobin in the peptic range, while mainly due to pepsinogen, is in part due to another, unidentified proteolytic enzyme. Results which follow will show that this proteolysis is in fact due to the nonenzymatic splitting of the proteins of the added plasma by the HCl used in the assay and is not due to another proteolytic enzyme.

Inactivation studies were done first on untreated plasma presumed to contain pepsinogen rather than pepsin. In keeping with the known resistance of pepsinogen to inactivation, no decrease in assayable peptic activity was produced by first alkalinizing the plasma to pH 11 and then returning it to pH 2 for assay; while boiling the plasma destroyed all traces of activity, temperature elevation to 70° C. for ten minutes did not decrease assayable activity. Ultraviolet (U.V.L.) irradiation (3,660 Å) at 25° C. for 36 hours did not decrease the peptic activity of the plasma.

Acidified ("activated") plasma, i.e, plasma in which acidification is presumed to have irreversibly converted all pepsinogen to pepsin,[9] showed the pH and temperature inactivation characteristics of a solution of crystalline pepsin[9]; it showed decreasing peptic activity at temperatures between 42° and 56° C. and was inactive above that temperature (Fig. 1) and also after alkalinization to pH 8 or over. Ultraviolet irradiation as noted with untreated plasma did not decrease peptic activity when acidified plasma was irradiated at 0° C. for 24 hours.

Except for the ultraviolet irradiation studies mentioned, the findings thus far were in keeping with the known inactivation data of pepsin and of pepsinogen in the crystalline state and constituted strong circumstantial evidence that the enzyme studied was pepsinogen.

Mirsky and associates[7] reported, however, that pH inactivation studies performed by them had shown a constant residue of activity after adequate pH inactivation studies, amounting to about 145γ (equivalent to about 2 P.U. per 100 ml. in the present study) of l-tyrosine per milliliter plasma in 24 hours, irrespective of the initial level of pepsinogen found. The very constancy of this residual "proteolytic activity" suggested that this was unlikely to be due to a naturally occurring proteolytic enzyme, and that it probably represented the lysis of some relatively constant protein constituent of the assay system. However, these workers[7] took into account in the blank the

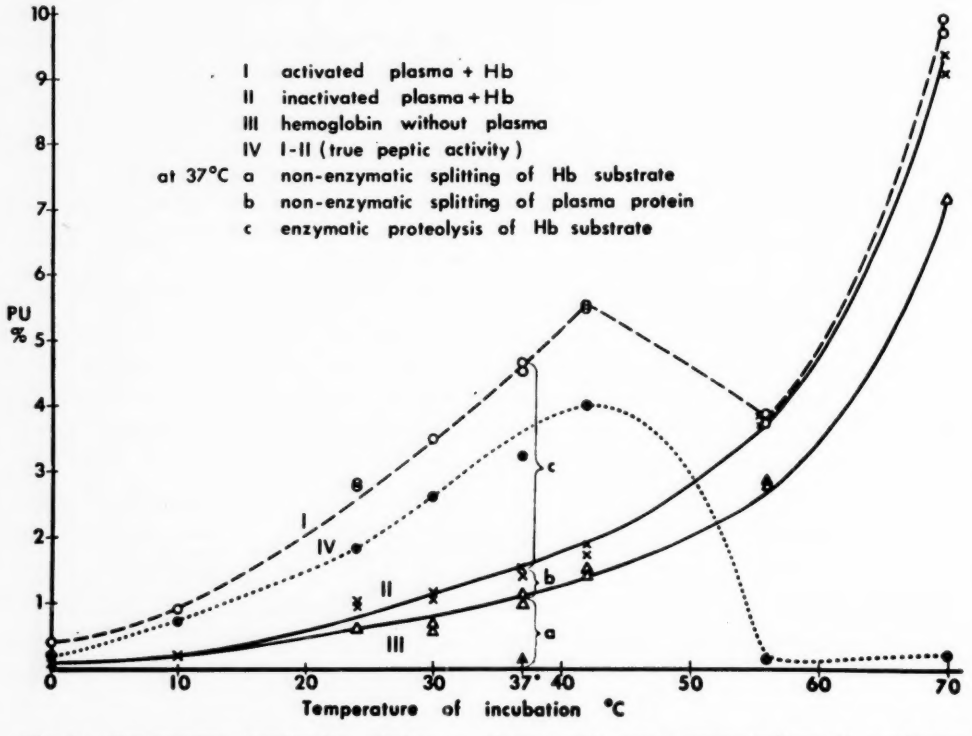

Fig. 1.—Temperature inactivation studies on plasma with small concentrations of pepsinogen indicating the presence of only one enzyme active at pH 1.85.

hydrolysis of the acidified hemoglobin substrate itself. This left the protein of the added plasma as the probable source of the relatively constant residual fraction of split products found in inactivated or "inactive" (postgastrectomy and pernicious anemia) plasmas. To test this hypothesis without denaturing the protein and thus making it unsusceptible to the acid hydrolysis, one sample of plasma of relatively low peptic activity was acidified with HCl to pH 1.85 to convert all the pepsinogen to pepsin, and then divided into three parts. One part was irradiated with U.V.L. at 0° C. for 24 hours, the second was alkalinized to pH 11 and then reacidified (inactivated plasma), and the third "activated plasma" assayed as such. The three fractions were then

assayed at pH 1.85 by the standard hemoglobin procedure for 24 hours at 0° 10°, 25°, 37°, 42°, 56°, and 72° C. Simultaneously, the hemoglobin substrate was incubated with 1 ml. of water at the same temperatures and pH to provide a blank for the inactivated plasma, where the latter in turn would serve as the blank for the active plasma. Fig. 1 will show that the proteolysis of the active plasma from which the proteolysis of the inactivated plasma is subtracted reacts to temperature changes as would be expected from a solution of pepsin; in the inactivated plasma the increase of tyrosine-like products of digestion was found to be parallel to that of the nonenzymatic breakdown of the hemoglobin, the difference in the two curves representing the nonenzymatic lysis of the protein in the plasma and the activity ascribed by Mirsky and associates[7] to a second proteolytic enzyme active in the acid range. The ultraviolet-irradiated plasma showed normal true peptic activity, but its protein was about half again as suspectible as pH-inactivated plasma to nonenzymatic acid hydrolysis at 70° C.

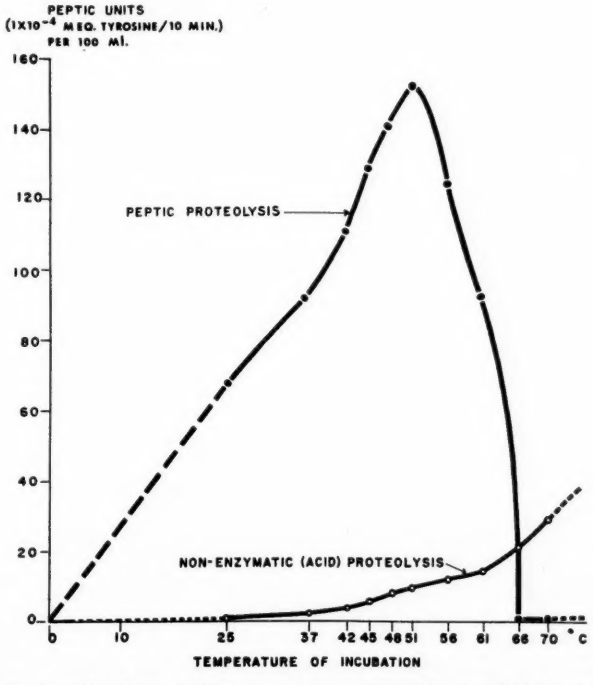

Fig. 2.—Temperature inactivation studies on plasma with high pepsinogen concentration. The lower curve is that of hydrolysis of the substrate without added plasma.

In addition to the above inactivation studies by pH and temperature changes on plasma of low peptic activity to show the acid hydrolysis of the plasma protein, temperature inactivation on one sample of plasma of very high peptic activity was done to determine more accurately the curve of temperature inactivation of the enzyme. As will be seen from Fig. 2, inactivation commences at temperatures above 51° C. and is complete at 66° C.

The differences noted between the minimum temperature of inactivation of pepsinogen in the plasmas of high and low enzyme content may be due to the difficulty of determining accurately such an end point at low concentrations of enzyme in plasma.

Distribution of the Enzyme in the Blood.—Pepsinogen can also be demonstrated in the red and white cells of the blood. Van Calcar[11] first described pepsinogen in both the plasma and in the leukocytes of the dog, supporting his findings by the disappearance of the enzyme from both after total gastrectomy.

Barnes,[2] more recently, was unable to demonstrate any proteolytic in either the polymorphonuclear leukocytes or the lymphocytes of the cat or the rabbit at pH below 3.0. I was unable to find any pepsinlike activity in the leukocytes of albino rats, whereas the plasma of these animals contained pepsinogen in concentrations approximating that of man.

Table II shows the distribution of peptic activity in human blood assayable at pH 1.9 ± 0.2. The white cells were obtained from the buffy coat of 20 ml. of heparinized blood centrifuged in narrow containers and resuspended in saline and assayed within 24 hours. The cell count in this suspension was about 600,000 per cubic millimeter. For the sake of comparison with other portions and cells of the blood, results are given as peptic units per 100 ml. of this suspension. The red cells were washed twice in normal saline and sedimented by centrifugation at 2,000 r.p.m.

TABLE II. PEPSINOGEN (P.U.%) IN VARIOUS FRACTIONS OF THE BLOOD

CASE	PLASMA	SERUM	WBC	RBC	WHOLE BLOOD	TIME AFTER GASTRECTOMY
A 1	55	55	142	73	89	–
2*	164	152	140	113	–	–
3*	216	212	–	–	–	–
4	28	–	68	–	–	–
5	12	–	183	–	–	–
6	40	–	–	–	50	–
B 7	43	–	–	–	153	1 day
8	0	–	21	–	–	2 days
9	0	–	130	–	–	3 days
10	0	–	103	–	–	3 days
11	0	–	55	–	–	7 days
12	4	–	±40	–	–	4 years

A=With intact stomachs.
B=After total gastrectomy.
*Uremic.

It will be noted that there is no difference between the pepsinogen concentration of plasma and of serum, whereas the concentration in the leukocytes tends to be much higher, though, with the crude methods employed for obtaining the white cell fraction, no reliable comparison can be made between the leukocyte and the red cell or plasma pepsinogen.

Sources of Pepsinogen in the Blood.—The disappearance of plasma pepsinogen after total gastrectomy has been described[7, 8, 11] and has indicated its gastric origin. The following results support and amplify these findings. At an operation on one patient who was undergoing *total gastrectomy,* blood was

taken simultaneously from a peripheral vein and from the left gastric vein as soon as the stomach was exposed, and again from a peripheral vein two and one-half hours after the stomach was removed, with the following results:

Before gastrectomy:	Peripheral blood	20 P.U.%
	Gastric vein blood	46 P.U.%
After gastrectomy:	Peripheral blood	0 P.U.%

Plasma pepsinogen levels were obtained on five other patients who had a total gastrectomy. Unfortunately, preoperative levels were obtained on only three of the six subjects, but microscopically none had complete gastric atrophy from which a pre-existing zero level might have been adduced (Table III).

TABLE III. DISAPPEARANCE OF PLASMA PEPSINOGEN (IN 5 OF 6 PATIENTS) WITHIN ONE TO SEVEN DAYS AFTER REMOVAL OF THE STOMACH (CASE 1 DESCRIBED IN THE TEXT)

CASE	PREOP.	POSTOPERATIVE							
		2 HR.	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS
1	20	0	–	–	–	–	–	–	–
2	16	12	6	–	–	–	–	–	0
3	–	–	–	–	0	–	–	–	–
4	–	–	–	–	0	–	–	–	–
5	19	–	0	0	0	0	–	–	–
6	–	–	43	–	–	–	–	–	5

The foregoing results indicate that the proteolytic enzyme in the plasma being described arises from the stomach. The complete disappearance in five of six cases further suggests that the enzyme being measured is probably wholly pepsinogen.

The presence of almost normal concentrations of pepsinogen in the white cells of blood with apeptic plasma following total removal of the stomach suggests either that the white cells retain and concentrate pepsinogen from the stomach or that the pepsinogen is manufactured in the white cells themselves. The latter premise should explain the finding of considerable, even if reduced, amounts of pepsinogen in the white cells in one patient four years after a total gastrectomy.

The Effects of Partial Gastrectomy.—The results of studies on four subjects undergoing Bilroth I partial gastric resection are summarized in Table IV.

TABLE IV. IMMEDIATE EFFECTS OF PARTIAL GASTRECTOMY ON PLASMA PEPSINOGEN

SITE OF ULCER	PREOP.	POSTOPERATIVE									
		1 HR.	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS	8 DAYS	9 DAYS
Gastric	23	13	25	30	30	60	9	11	11	17	21
Duodenal	59	53	41	52	52	–	–	–	–	–	–
Duodenal	85	67	78	63	63	–	–	–	–	–	–
Duodenal	31	–	26	63	61	31	47	–	–	–	13

The figures in Table IV show that the fundus is capable of maintaining and even of increasing the plasma pepsinogen concentration after the removal of the lower half of the stomach, and thus the fundus may normally be the

TABLE V. COMPARISON OF MEANS OF PLASMA PEPSINOGEN CONCENTRATION AND GASTRIC PEPSIN OUTPUT PER HOUR COLLECTED SIMULTANEOUSLY*

NAME	TIME OF EXPERIMENT	PLASMA PEPSINOGEN P.U.% (MEAN)	GASTRIC PEPSIN P.U./H.R. (MEAN)	G.P./P.P.
		A. Low Secretors		
C. S.	7 days	20.5	81,000	3,951
	21 days	17.5	74,300	4,245
	12 hours	17.6	78,000	4,431
	14 hours	8.9	62,600	7,033
	13 hours	12.6	78,000	6,190
	14 hours	15.0	81,500	5,433
H. P.	7 days	6.7	34,500	5,160
	21 days	11.5	30,700	2,720
	12 hours	11.5	48,400	4,030
	14 hours	10.3	12,300	1,200
	14 hours	9.4	13,000	1,300
	13 hours	6.9	59,500	8,500
C. F.	15 hours	11.0	79,300	7,209
	13 hours	11.3	101,000	8,938
	13 hours	10.5	83,000	7,874
	13 hours	13.3	87,000	6,541
	13 hours	15.6	86,330	5,533
	13 hours	9.4	101,580	11,236
O. G. W.	7 days	20.0	82,000	4,100
	6 days	18.0	77,500	4,305
	13 hours	13.3	50,950	3,831
	13 hours	13.3	46,000	3,458
	13 hours	16.5	54,400	3,296
S. P.	12 hours	8.8	53,000	6,022
	13 hours	7.5	38,540	5,138
	13 hours	11.3	60,150	5,323
	13 hours	14.1	54,323	3,853
	14 hours	15.8	50,250	3,180
M. F.	12 hours	11.0	71,160	6,469
	13 hours	7.8	56,600	7,256
	13 hours	10.4	61,900	5,951
	14 hours	12.4	83,000	6,690
J. K.	11 hours	10.2	65,600	6,431
	13 hours	8.5	45,500	5,352
	13 hours	12.0	103,000	8,580
	13 hours	16.0	72,230	4,514
D. B.	7 days	12.7	37,000	2,913
	6 days	10.4	36,000	3,461
	12 hours	11.7	88,000	7,521
B. D.	13 hours	14.2	56,000	3,943
	13 hours	17.8	37,300	2,095
H. B.	16 days	10.4	48,000	4,615
	12 hours	16.6	54,000	3,253
H. W.	7 days	17.0	59,000	3,470
	21 days	16.2	71,600	4,419
M. B.	14 hours	10.8	82,000	7,592
M. Dil.	13 hours	18.6	56,800	3,047
	Means	11.7	62,400	5,333

*Correlation coefficient between plasma pepsinogen and gastric pepsin in all subjects $r=+.603$ $t=5.53$ $p<.001$. Difference between pepsin output in low and high secretors "t"$=5.98$ $.0001<p<.001$ Between ratios high and low secretors $t=8.18$ $.0001<p<.001$.

TABLE V.—CONT'D

NAME	TIME OF EXPERIMENT	PLASMA PEPSINOGEN P.U.% (MEAN)	GASTRIC PEPSIN P.U./H.R. (MEAN)	G.P. / P.P.
		B. High Secretors		
J. W.	11 hours	12.1	138,000	11,404
	14 hours	14.6	146,000	10,000
	14 hours	14.3	173,000	12,079
	13 hours	10.0	50,100	5,010
	13 hours	10.3	80,000	7,766
	13 hours	8.3	100,000	12,000
R. C.	13 hours	14.2	154,000	10,845
	13 hours	5.9	90,000	15,254
	13 hours	12.3	107,000	8,713
	13 hours	16.8	101,000	6,030
D. C.	7 days	18.8	183,000	9,734
	7 days	20.6	228,000	11,067
	13 hours	20.8	289,000	13,895
	13 hours	17.5	221,000	12,260
W. D.	7 days	12.0	119,000	9,916
	7 days	15.2	156,000	10,260
A. C.	13 hours	11.1	119,000	10,720
	Means	13.8	143,700	10,400

main source of the plasma pepsinogen. The absence of an immediate postoperative rise suggests that mechanical handling of the stomach does not play a part in the later rises of pepsinogen seen in two of the above subjects.

The Relation Between Plasma Pepsinogen and Gastric Pepsin.—In eight normal subjects daily plasma pepsinogen estimations and thrice weekly 1-hour basal gastric analyses were performed for 13 to 28 days. The mean values in the first week were used to determine the ratios between the plasma pepsinogen concentration (P.U. per 100 ml.) and the 1-hour gastric pepsin output, and these were compared with the ratios in the subsequent period of the study. In five of these subjects and in ten other normal subjects from one to six one-day experiments were done in which the mean of the two-hourly plasma pepsinogen estimations was compared to the mean hourly output of gastric pepsin over the 12 to 14 hours of the study; specimens of gastric juice were obtained by continuous suction over the whole period of the study and divided into half-hourly samples and assayed separately for pepsin. Because of the specimen-to-specimen variability of fasting gastric pepsin output in some of our subjects, the means of the gastric pepsin and of plasma pepsinogen were compared rather than at any one particular moment when each plasma pepsinogen estimation was done.

The results detailed in Table V indicate a significant positive correlation between the mean hourly gastric pepsin output and the plasma pepsinogen concentration in all subjects ($r = +0.603$) with a mean ratio of 6237 (SD ± 3503).

As will be noted from Table V there were fluctuations in both the gastric and the plasma pepsinogen from day to day in the same subjects, but the

ratio between the two remained relatively constant. The significance of these small but important covariations will be discussed later. It will be further noted that of the 18 subjects the last 5 in Table V had distinctly higher ratios than the others ("t" = 8.18, $.0001<p<.001$). This difference was accounted for by a higher output of gastric pepsin in this group of normal subjects than in the rest ($t = 5.978$, $.0001<p<.001$).

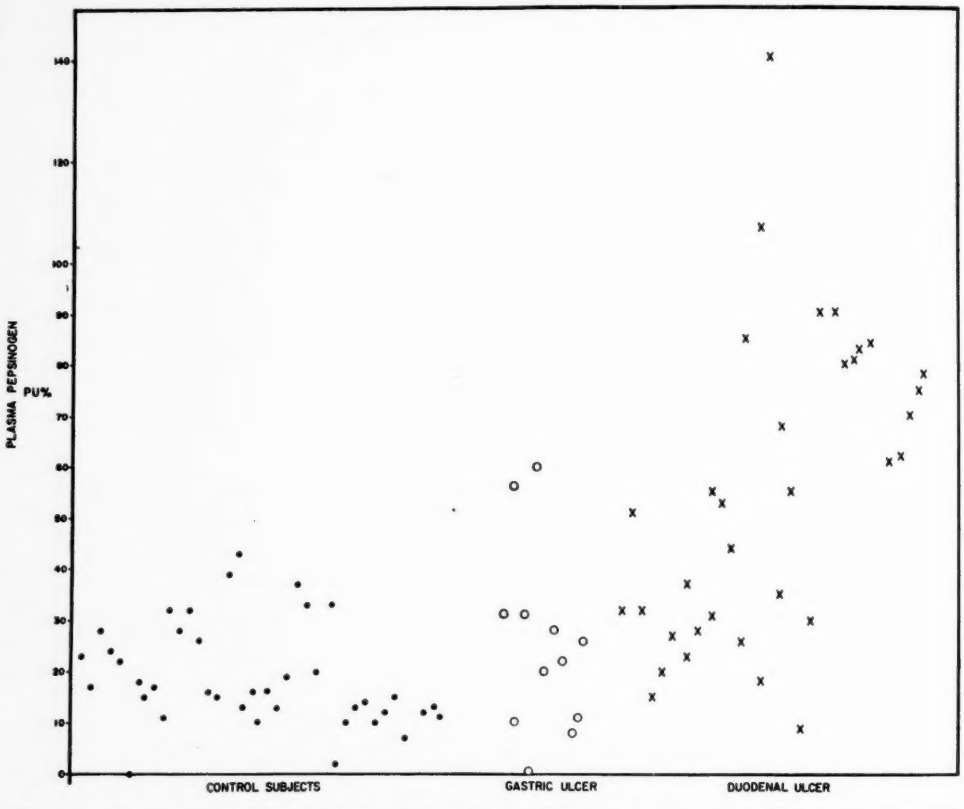

Fig. 3.—Plasma pepsinogen levels (peptic units per 100 ml.) in normal controls and in gastric and duodenal ulcer subjects.

Poor correlation existed between concentrations of gastric pepsin and plasma pepsinogen because the concentration of pepsin in the gastric juice rarely exceeds 3,000 P.U. per milliliter and never exceeds 3,500 P.U. per milliliter. The increased output of gastric pepsin in hypersecretors, therefore, is mainly accounted for the increased volume of gastric secretions, and it is total output of pepsin and not concentration which characterizes the pepsin secretion in hypersecretors.

Plasma Pepsinogen in Peptic Ulcer.—Plasma pepsinogen levels were determined in 45 normal persons, 37 men with duodenal ulcers, and 15 patients (2 women) with gastric ulceration (Table VI). Two of the 15 patients with gastric ulcers and about 60 per cent of the patients with duodenal ulcers had significantly elevated levels of plasma pepsinogen. (Fig. 3.)

TABLE VI. PLASMA PEPSINOGEN LEVELS IN CONTROLS AND GASTRIC AND DUODENAL ULCER SUBJECTS

GROUP	NO.	RANGE (P.U.%)	MEAN (P.U.%)	S.D.	COMPARE TO	STATISTICAL TESTS "t"	P
A. Control	45	0-43	17.2	± 9.1	B	2.18	<.05
B. Gastric Ulcer	12	0-60	25.4	±17.4	C	2.916	.01 -.05
C. Duodenal Ulcer	37	31-210	60.4	±39.5	A	7.04	.001-.0001

These findings agree with the findings of Mirsky and associates[8] who reported on much larger and more comprehensive groups of normal and duodenal ulcer subjects. The near normal levels in gastric ulcer subjects correspond to similar findings in urinary pepsinogen excretion[14] as do the findings in the duodenal ulcer group.

The levels of plasma pepsinogen in six patients with uremia were raised, and ranged from 41 to 216 P.U. per 100 ml., paralleling the rise of the blood urea and confirming similar findings in animals by Loeper and Debray.[5] In both the control and the ulcer subjects in whom abnormal levels were found, blood urea and plasma creatinine levels were found to be normal. Uremia should be ruled out whenever an abnormal level of plasma pepsinogen is found.

DISCUSSION AND CONCLUSIONS

The proteolytic enzyme found in the blood which is active at acid pH is not pepsin itself,[3, 7, 12] but is derived from the stomach as evidenced by the complete disappearance of the enzyme from the plasma after total gastrectomy and by the higher concentrations found in the gastric vein than in the peripheral blood. The indications that the enzyme as assayed is pepsinogen only is circumstantial; but, with the published evidence on its inactivation behavior[7] and the evidence presented here, one may conclude with some certainty that it could only be pepsinogen.

While no teleological significance can be ascribed to the presence of this enzyme in the leukocytes, the evidence seems to point to the white blood cells as a possible site of extragastric pepsinogen manufacture. The amounts found in the leukocytes are very small, compared to those elaborated by the stomach and should in no way interfere with the general premise that the stomach is responsible for all but a minute fraction of the pepsinogen found in the plasma and the urine.

The relatively narrow range of the ratio between the plasma pepsinogen concentration and the gastric pepsin output appears to afford a reasonable indirect and yet simple means of assessing gastric pepsin secretion. One conclusion and one surmise can be drawn from the material presented in this regard. First, the relatively small variations in any one individual present similar changes in the amount of pepsin being secreted by the stomach, not so much at any particular moment, but over a period of several hours or days when the mean output measured in the gastric juice is more representative of the output of the stomach. It should be emphasized that these studies represent

only basal and near-basal gastric secretion. Maximal stimulation of gastric pepsin secretion will in fact actually decrease the concentration of plasma pepsinogen,* possibly by withdrawing the pepsinogen from the cell/capillary contact wall (the "diffusing" end) to the luminal or "secreting" end of the cell.

The surmise that can be drawn from the available evidence is that the low levels of plasma pepsinogen found in patients with pernicious anemia[3, 8] and the high levels found in most patients with duodenal ulceration represent not so much the actual amounts of pepsin being secreted by the stomach, as the total gastric peptic cell mass or the "peptic potential" of the stomach; as stated above, the minor variations from the mean level in any one individual at any one time represent the changes in secretion of pepsin by the stomach under basal or near-basal conditions. It is well known that the stomach does not secrete acid or pepsin at anywhere near maximal levels under ordinary circumstances and that the so-called basal secretory levels may, therefore, vary considerably at different times (Table V), whereas the maximal secretion by that stomach should be reproducible on repeated testing. That has been proved for the acid secretion of the stomach by Kay,[4] who found that individuals differed in their total acid-secreting cell mass, patients with duodenal ulcers secreting more on account of an increased cell mass. The relative constancy of the plasma pepsinogen levels in any one individual, whatever the level of pepsinogen concentration,[7] further supports the contention that the plasma pepsinogen represents the peptic potential of that subject's stomach. This may afford a simple means for the long-term study of the effects of peptic ulceration on other diseases of the stomach.

As with the gastric acid and with urinary pepsinogen excretion[14] the plasma pepsinogen cannot be used as the sole means of diagnosis of upper gastrointestinal disease. The facility with which specimens can be collected and assayed, however, makes it at least as valuable as the other two measurements of gastric function, but it should be again emphasized that these tests can and should only be used as auxiliaries to the established methods of diagnosis of gastric lesions.

SUMMARY

1. Evidence on the proteolytic enzyme active at pH 1-3 found in human blood is presented to show that there is but one enzyme, most likely pepsinogen, derived mainly, if not wholly, from the stomach.

2. Pepsinogen is found in the plasma and the red and white cells of human blood. That found in the white cells may be manufactured endogenously.

3. It is suggested that the mean level plasma pepsinogen in any one individual represents the total gastric peptic cell mass or "peptic potential" of the stomach, and it has been shown that the small variations about the mean in an individual represent concomitant changes in the basal or near-basal secretion of pepsin by the stomach.

4. Plasma pepsinogen concentrations were found to be normal in 13 of 15 patients with gastric ulcer and elevated in about 60 per cent of patients with

*Unpublished observations.

duodenal ulceration and in patients with uremia. The estimation of plasma pepsinogen may form a useful adjunct to other methods of studying gastric secretion.

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ACQUIRED HEMOLYTIC ANEMIA AND VIREMIA

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IN LIGHT of the work of previous investigators[1-3] who reported the isolation of the viruses of Newcastle disease and herpes and several unknown viral agents from the blood of patients with acquired hemolytic anemia, it was of interest to attempt to isolate viruses from the blood and tissues of a series of patients with acquired hemolytic anemia[4] under active study in the Medical Clinics of the Strong Memorial Hospital.[5]

MATERIALS AND METHODS

1. *Patients.*—Eight patients with acquired hemolytic anemia in various phases of activity were studied. The specimens obtained and concurrent clinical and laboratory findings in these patients are presented in Table I. Data on these patients will be presented in greater detail in another publication.[5]

2. *Methods for Virus Isolation.*—The whole clotted blood or splenic tissue was stored at -40° C. until tested. All of the specimens but two spleens (E. H. and A. F.) were tested soon after collection. At the time of test, the specimens were thawed and ground with alundum in a mortar to prepare a suspension in beef heart infusion broth. After the addition of 100 units of penicillin and of streptomycin per milliliter to these suspensions they were injected into embryonated eggs by various routes. The volumes injected, route employed, and age of embryos was as follows: 0.1 ml. into the amniotic sac of 10-day-old chick embryos; 0.2 ml. into the allantoic sac of 8-day-old embryos; 0.5 ml. into the yolk sac of 7-day-old embryos.

The injected eggs were incubated at 35° C. and examined daily for four days at which time the amniotic, allantoic, and yolk fluids were harvested and tested with a 0.5 per cent suspension of chicken erythrocytes for the presence of hemagglutinating viruses. Three serial blind passages were carried out on these fluids, using the three respective routes of inoculation described, and tests were done on the fluids from each passage for the presence of hemagglutinating agents.

The fluids and a suspension of yolk sac tissues from the third blind passage were inoculated (0.2 ml.) on the chorioallantoic membranes of 11-day-old chick embryos. These embryonated eggs were incubated at 35° C. for 5 days, at which time the corioallantoic membranes were removed from the eggs, placed in sterile saline (0.85 per cent) in Petri dishes, and examined with a dissecting microscope for the presence of lesions.

RESULTS

No evidence was obtained for the presence of viral agents in the whole clotted blood or splenic tissue through death of the embryos, presence of visible changes in the chick embryos, hemagglutination tests on egg fluids or development of visible lesions on the chorioallantoic membranes on any passage inoculation in these studies.

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TABLE I. CLINICAL AND LABORATORY FINDINGS ON PATIENTS WITH ACQUIRED HEMOLYTIC ANEMIA AT TIME SPECIMENS WERE TAKEN FOR VIRUS ISOLATION

CASE	ASSOCIATED DISEASE	SPECIMEN FOR VIRUS ISOLATION	HEMATOCRIT (%)	RETICULOCYTES (%)	BILIRUBIN MG./100 ML.	DIRECT ANTIGLOBULIN (COOMBS) TEST	"AUTOANTIBODY" IN SERUM*	AUTOAGGLUTINATION†
R. C.	Chronic lymphocytic leukemia	Blood	41	3.3	<1.0	Negative (previously positive)	0 (previously present)	0
E. H.	None	Blood	20	5.2	2.2	Positive	Present	+++
		Spleen	21	11.8	-	Positive	Present	+++
H. M.	None	Blood	12	33.0	2.4	Positive	Present	+
D. M.	Chronic lymphocytic leukemia	Blood, Spleen	26	1.4	-	Positive	0	±
A. F.	None	Spleen	19	4.1	15.8	Positive	Present	++
W. T.	None	Blood	27	10.0	-	Positive	Present	+
		Spleen	42.5	0.7	-	Positive	Present	+
S. T.	None	Blood	24	36.0	2.8	Positive	Present	++
		Blood	25	44.0	-	Positive	Present	++
R. M.	None	Blood	13	4.0	1.0	Negative	Present	++++

*"Autoantibody" demonstrated at 37° C. against both autogenous and normal red cells by indirect antiglobulin test, or by agglutination of trypsin-treated red cells or by both techniques.

†Macroscopically visible agglutination of red cells in oxalated blood sample immediately after withdrawal from peripheral vein. Range in degree of agglutination from 0 to ++++ (marked).

DISCUSSION

The results of these experiments indicate that it is unlikely that viruses such as Newcastle disease, influenza, mumps, psittacosis, herpes, certain pox viruses, and closely related agents occurred in the blood and splenic tissues of these patients since the techniques employed are probably adequate for the demonstration of these viruses. Furthermore, the serial blind passages should have permitted the recovery of viruses that might have been "masked" in the initial blood or tissue suspensions due to the presence of antibody or inhibiting materials.

It does not appear that viremia due to these viruses is a common finding in patients with acquired hemolytic anemia as suggested by other investigators[1-3] even when such patients are in a stage of hemolytic crisis.

SUMMARY

No evidence of viremia was obtained from the inoculation and serial passage in embryonated eggs of suspensions of whole blood and splenic tissue obtained from eight patients with acquired hemolytic anemia even though such patients were in a stage of active hemolysis.

I wish to express my appreciation to Drs. Lawrence Young and Gerald Miller for providing the whole blood and splenic tissues and the clinical and laboratory data from these patients and to acknowledge the technical assistance of Mr. John Bader.

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IN VITRO ACTIVITY AGAINST MICROCOCCUS PYOGENES OF VARIOUS COMBINATIONS OF ANTIMICROBIAL AGENTS

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THE increasing resistance of *Micrococcus pyogenes* to antimicrobial agents and the severe, often fatal, infections caused by these resistant bacteria have been described by other investigators.[1-5] The purpose of this report is to present the results of in vitro studies in which the bacteriostatic and bactericidal activity against *Micrococcus pyogenes* of various combinations of antibiotics have been determined.

Severe fulminating infections due to *M. pyogenes* should always be treated initially with more than one drug because no single antimicrobial agent has proved invariably effective against infections due to this species.[5] Combinations of antimicrobial drugs also should be used in certain chronic infections which body defenses cannot penetrate and in which a bactericidal effect may be necessary for cure. A third indication for the use of combinations of antimicrobial agents in infections due to *M. pyogenes* is in cases in which a potentiating or additive effect may be necessary. The results reported may be of some help to the clinician in the selection of a combination of agents when one of these three indications is encountered.

MATERIALS AND METHODS

All strains of *M. pyogenes* that were tested were coagulase positive and were isolated from patients in the wards of the Milwaukee County General Hospital. The sensitivity of the strains to the various antibiotics was determined by the tube dilution method.[6] In this method 0.5 ml. aliquots of a 1 to 100 dilution of an eighteen-hour growth of the bacteria in dextrose broth is added to decreasing concentrations of the antibiotics dissolved in 0.5 ml. of distilled water. Twofold concentrations ranging from 0.19 to 100 μg per milliliter of drugs are used. The end point is read after eighteen-hour incubation at 37° C. and is the lowest concentration of drug that will prevent visible growth. The bactericidal modification of this test is made by subculturing a 0.1 ml. aliquot from each of the clear tubes in the original test into 10 ml. of dextrose broth.[6] The bactericidal end point is the lowest concentration of drug in which these aliquots show no growth.[6] When combinations of antibiotics are tested, a 0.5 ml. aliquot of each antibiotic is placed in each tube and 1 ml. of a 1 to 100 dilution of an eighteen-hour growth of the bacteria is added. Recently, some investigators have raised a question regarding the reproducibility of the tube dilution method.[7, 8] These questions may be due to the fact that these workers have used relatively few organisms as an inoculum.[7] However, during the past six years we have found that this method, when it is performed as outlined previously, is not only consistent but also that it correlates very well with the results of therapy as we have personally observed it at the bedside.[9-12] Multiple determinations were performed on two strains of *M. pyogenes* to test further the reproducibility of the method (Table I). In 18 of the 20 determinations comparable results were obtained.

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TABLE I. THE RESULTS OF TEN DETERMINATIONS MADE WITH EACH OF TWO STRAINS OF MICROCOCCUS PYOGENES AGAINST THE SAME ANTIBIOTICS IN ORDER TO TEST THE REPRODUCIBILITY OF THE TUBE DILUTION METHOD

TEST NUMBER	MINIMAL BACTERIOSTATIC INHIBITORY CONCENTRATION		MINIMAL INHIBITORY CONCENTRATION OF THE COMBINATION OF PENICILLIN AND ERYTHROMYCIN	RESULT
	PENICILLIN	ERYTHROMYCIN		
Strain 1— 1	3	50	1.5*	P†
2	3	50	1.5	P
3	3	50	1.5	P
4	3	50	1.5	P
5	3	100	1.5	P
6	3	100	1.5	P
7	3	100	1.5	P
8	3	>100	1.5	P
9	3	>100	1.5	P
10	3	>100	1.5	P
Strain 2— 1	6	>100	6	N‡
2	12	>100	6	P
3	12	>100	12	A§
4	25	>100	12	P
5	25	>100	12	P
6	25	>100	12	P
7	50	>100	12	P
8	100	>100	12	P
9	25	>100	12	P
10	25	>100	12	P

*1.5 means 0.75 μg/ml. of penicillin and 0.75 μg/ml. of erythromycin mixed together.
†P=Potentiation.
‡N=No effect.
§A=Additive effect.
(See text of Materials and Methods for our exact definitions of these terms.)

TABLE II. THE PERCENTAGE OF 100 STRAINS OF M. PYOGENES ISOLATED DURING 1954 FROM PATIENTS ON THE WARDS OF THE MILWAUKEE COUNTY GENERAL HOSPITAL THAT WERE SENSITIVE TO THE ANTIMICROBIAL AGENTS USED IN THIS STUDY

DRUG	INHIBITORY CONCENTRATION OF DRUG CONSIDERED THE UPPER LIMIT OF CLINICAL SENSITIVITY IN μG/ML.*	PER CENT OF STRAINS SENSITIVE
Penicillin	1.5	13
Chlortetracycline	3	56
Oxytetracycline	3	53
Chloramphenicol	25	94
Streptomycin	1.5	30
Bacitracin	3	52
Erythromycin	3	72
Nitrofurantoin	12	92
Tetracycline	3	53

*These values were arbitrarily selected on the basis of our experience with the correlation of sensitivity tests and clinical results.

The drugs used in combination were selected because singly they were the most effective agents against the strains of *M. pyogenes* isolated in the Milwaukee County General Hospital (Table II). All possible combinations were not tested. Each combination of antimicrobial agents was tested against ten strains of *M. pyogenes* and bacteriostatic and bactericidal end points were determined. The results of combining the drugs were defined in the following manner:

1. *No effect:* It was assumed that the addition of one antimicrobial agent to another had no effect if the end point of the most effective agent remained the same. For instance, if a concentration of 3 μg per milliliter of chlortetracycline inhibited a strain and a concentration of 3 μg per milliliter of chlortetracycline and 3 μg per milliliter of streptomycin also inhibited the same strain, it was felt that the addition of the streptomycin had no effect on the activity of the chlortetracycline.

2. *Additive effect:* It was assumed that an additive effect was demonstrated when the total concentration of antimicrobial drugs in the inhibitory combination was equal to the concentration of the most effective drug when it was used alone. For instance, an additive effect would be reported if 3 μg per milliliter of chlortetracycline was inhibitory and if 1.5 μg per milliliter of chlortetracycline and 1.5 μg per milliliter of streptomycin mixed together were inhibitory.

3. *Potentiation:* It was assumed that potentiation had occurred when the combination of two drugs resulted in inhibition of a strain by a lower concentration of drugs than was necessary when either of the drugs was used alone. For instance, potentiation would be reported if a strain of bacteria was inhibited by 6 μg per milliliter of chlortetracycline and 6 μg per milliliter of streptomycin and was inhibited by 0.38 μg per milliliter of each drug when they were combined.

4. *Inhibition:* It was assumed that inhibition had occurred when the end point of a drug was raised when a second drug was added to it. For instance, if a strain was inhibited by 3 μg per milliliter of chlortetracycline and the end point was raised to 6 μg per milliliter of chlortetracycline when 6 μg per milliliter of streptomycin also was present, it would be assumed that the streptomycin inhibited the action of the chlortetracycline. Erythromycin, chlortetracycline, nitrofurantoin, chloramphenicol, bacitracin, tetracycline, penicillin, and streptomycin were the antibiotics tested in the various combinations.

RESULTS

The bacteriostatic results of combining the various antimicrobial drugs against *M. pyogenes* are presented in Table III. There was no effect in 40 determinations, an additive effect in 49 determinations, potentiation in 20 determinations, and inhibition in 7 instances. Thus, in 69 of 116 determinations, an advantage in bacteriostatic activity was gained by the use of a combination of drugs. The two combinations that appeared most effective when judged

TABLE III. BACTERIOSTATIC RESULTS OF COMBINING ANTIBIOTICS AGAINST STRAINS OF M. PYOGENES

COMBINATION	NO. OF STRAINS	NO EFFECT	ADDITIVE EFFECT	POTENTIATION	INHIBITORY
Erythromycin and chlortetracycline	10	4	3	3	0
Nitrofurantoin and chloramphenicol	10	1	9	0	0
Erythromycin and chloramphenicol	10	3	6	0	1
Erythromycin and nitrofurantoin	10	2	5	1	2
Chloramphenicol and bacitracin	10	0	7	2	1
Erythromycin and penicillin	10	4	3	3	0
Chlortetracycline and streptomycin	10	6	2	1	1
Erythromycin and streptomycin	10	6	2	1	1
Bacitracin and nitrofurantoin	8	3	5	0	0
Chlortetracycline and tetracycline	8	2	3	3	0
Chlortetracycline and bacitracin	10	6	1	2	1
Erythromycin and tetracycline	10	3	3	4	0
Total	116	40	49	20	7

by bacteriostatic activity were chloramphenicol and bacitracin, and chloramphenicol and nitrofurantoin. In both combinations an additive or potentiating result was obtained against 9 of the 10 strains. The 3 combinations that showed neither an additive nor a potentiating effect in over one-half of the 10 strains against which they were tried were chlortetracycline and bacitracin, erythromycin and streptomycin, and chlortetracycline and streptomycin (Table III). Fig. 1 shows more graphically some examples of the potentiating and inhibiting effects that resulted from the combining of the antimicrobial drugs. The magnitude of the bacteriostatic potentiation and inhibition that was demonstrated is presented in Table IV. The number of

TABLE IV. THE MAGNITUDE OF BACTERIOSTATIC INHIBITION AND POTENTIATION EXHIBITED BY THE COMBINATIONS OF ANTIMICROBIAL DRUGS

The factor* by which the minimal inhibitory bacteriostatic concentration was reduced by the use of a combination of drugs	Number of times this degree of potentiation occurred
2	4
4	13
8	3
16	
The factor† by which the minimal inhibitory bacteriostatic concentration was raised by the use of a combination of drugs	**Number of times this degree of inhibition occurred**
2	5
4	1
8	1
16	

*For instance, if 12 μg/ml. of chlortetracycline was inhibitory and 1.5 μg/ml. of each chlortetracycline and streptomycin or a total antibiotic concentration of 3 μg/ml. was inhibitory, the factor would be 4.

†Conversely if 1.5 μg/ml. of chlortetracycline was inhibitory and if 6 μg/ml. of chlortetracycline and 6 μg/ml. of streptomycin were necessary for inhibition, 4 times more chlortetracycline was needed and therefore the inhibition factor would be 4.

times each drug was involved in a combination that resulted in no effect, an additive effect, potentiation, and inhibition is presented in Table V. No one drug emerges as far superior to the others as an agent that promotes bacteriostatic potentiation, but erythromycin, chloramphenicol, and nitrofurantoin appear to have the best record in this respect.

The bactericidal results of combining the various antimicrobial drugs against *M. pyogenes* are presented in Table VI. There was no effect in 38

TABLE V. NUMBER OF TIMES EACH DRUG WAS INVOLVED IN A BACTERIOSTATIC COMBINATION THAT HAD NO EFFECT, AN ADDITIVE EFFECT, POTENTIATION, OR INHIBITION

DRUG	TOTAL NO. OF TIMES DRUG WAS USED	N*	A†	P‡	I§
Erythromycin	60	22	21	12	5
Chlortetracycline	38	20	8	8	2
Chloramphenicol	30	4	21	2	3
Nitrofurantoin	28	6	18	2	2
Bacitracin	28	9	13	4	2
Penicillin	10	4	3	2	1
Streptomycin	20	12	4	2	2
Tetracycline	20	8	5	7	0

*N—No effect.
†A—Additive effect.
‡P—Potentiation.
§I—Inhibition.
(See Materials and Methods for definitions of these terms.)

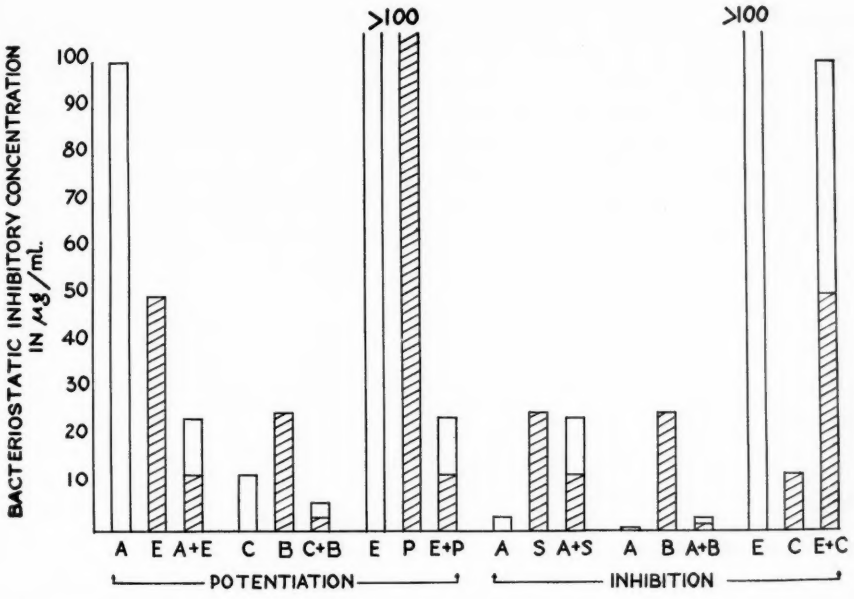

Fig. 1.—Examples of bacteriostatic potentiation and inhibition exhibited by various combinations of antimicrobial drugs. *A*, Chlortetracycline; *E*, erythromycin; *C*, chloramphenicol; *B*, bacitracin; *P*, penicillin; *S*, streptomycin.

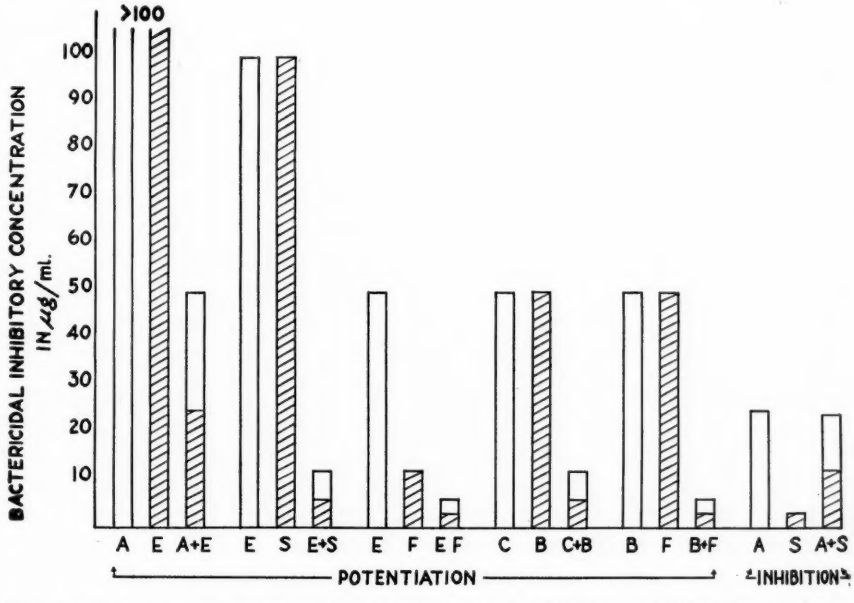

Fig. 2.—Examples of bactericidal potentiation and inhibition exhibited by various combinations of antimicrobial drugs. *A*, Chlortetracycline; *E*, erythromycin; *S*, streptomycin; *F*, nitrofurantoin; *B*, bacitracin.

determinations, an additive effect in 37 determinations, potentiation in 26 determinations and inhibition in 17 instances. A bactericidal advantage was gained by the use of a combination of drugs in 63 of the 118 tests. The two most advantageous combinations from a bactericidal point of view appeared to be erythromycin and chlortetracycline, and erythromycin and nitrofurantoin. In both of these combinations an additive or potentiating effect was obtained against 8 of the 10 strains. The least advantageous combinations from a bactericidal point of view were chlortetracycline and bacitracin, and chlortetracycline and streptomycin. No effect or inhibition was noted with these combinations eight and seven times, respectively.

TABLE VI. BACTERICIDAL RESULTS OF COMBINING ANTIBIOTICS AGAINST M. PYOGENES

COMBINATION	NO. OF STRAINS	NO EFFECT	ADDITIVE EFFECT	POTENTIATION	INHIBITION
Erythromycin and chlortetracycline	10	2	6	2	0
Nitrofurantoin and chloramphenicol	10	2	6	0	2
Erythromycin and chloramphenicol	10	5	2	1	2
Erythromycin and nitrofurantoin	10	2	4	4	0
Chloramphenicol and bacitracin	10	4	2	4	0
Erythromycin and penicillin	10	4	3	2	1
Chlortetracycline and streptomycin	10	3	2	1	4
Erythromycin and streptomycin	10	3	1	4	2
Bacitracin and nitrofurantoin	8	2	3	1	2
Chlortetracycline and tetracycline	10	3	4	3	0
Chlortetracycline and bacitracin	10	5	1	1	3
Erythromycin and tetracycline	10	3	3	3	1
Total	118	38	37	26	17

Fig. 2 shows examples of bactericidal potentiation and inhibition that resulted from some of the combinations. The magnitude of the bactericidal potentiation and inhibition that occurred is shown on Table VII. The number of times each drug was involved in a combination that exhibited no bactericidal effect, an additive effect, potentiation, or inhibition is shown in Table VIII. As in the case of bacteriostatic activity, no one drug emerged as markedly superior as a promoter of increased bactericidal activity and in certain instances each drug caused a potentiating effect. Similarly, bactericidal inhibition was caused by each drug in at least one instance.

DISCUSSION

These results confirmed the early conclusion of Spicer[13] who, in 1950, stated that when antimicrobial agents are combined, either potentiation or inhibition could occur. However, our results showed the magnitude and frequency of both additive and potentiating effects to be far greater than the

TABLE VII. THE MAGNITUDE OF BACTERICIDAL INHIBITION AND POTENTIATION EXHIBITED BY THE COMBINATION OF ANTIMICROBIAL DRUGS

The factor* by which the minimal inhibitory bactericidal concentration was reduced by the use of a combination of drugs	Number of times the degree of potentiation occurred
2	1
4	17
8	5
16	3
The factor by which the minimal inhibitory bactericidal concentration was raised by the use of a combination of drugs	**Number of times the degree of inhibition occurred**
2	13
4	3
8	
16	2

*See footnote to Table III.

TABLE VIII. NUMBER OF TIMES EACH DRUG WAS INVOLVED IN A BACTERICIDAL COMBINATION THAT HAD NO EFFECT, AN ADDITIVE EFFECT, POTENTIATION, OR INHIBITION

DRUG	TOTAL NO. OF TIMES DRUG WAS USED	N*	A*	P*	I*
Erythromycin	60	19	19	16	6
Chlortetracycline	40	13	13	7	7
Chloramphenicol	30	11	10	5	4
Nitrofurantoin	28	6	13	5	4
Bacitracin	28	11	6	6	5
Penicillin	10	4	3	2	1
Streptomycin	20	6	3	5	6
Tetracycline	20	6	7	6	1

*See Table IV for explanation of letters.

magnitude and frequency of the inhibitory effects that resulted from combining the antimicrobial drugs (Tables III to VI). The most advantageous combinations of drugs were not restricted to those in which a so-called bacteriostatic and bactericidal drug were combined. For example, the combinations of chlortetracycline and tetracycline and of chlortetracycline and erythromycin which consist of two bacteriostatic agents were superior in both bacteriostatic and bactericidal activity to the combination of chlortetracycline which is bacteriostatic and streptomycin which is bactericidal.[14, 15] Thus, in specific cases, certain combinations of antibiotics should not be neglected for in vitro studies merely because both drugs primarily are bacteriostatic. In this respect, our results did not support the conclusions of Jawetz and Gunnison[16] and Rantz and Randall[17] that the combination of primarily bacteriostatic agents offer little advantage. In the latter case this may be due to the fact that chloramphenicol and oxytetracycline was the only combination tested.[17] Furthermore, our findings indicated that the two least advantageous combinations from a bactericidal standpoint consisted of one bacteriostatic and one bactericidal drug (chlortetracycline and streptomycin, and chlortetracycline and bacitracin). This should further serve to emphasize that in problem cases, the causative strain should be tested in vitro against all possibilities rather than just against arbitrarily grouped classes of antibiotics.[16]

It is our opinion that these results should not encourage the routine use of combinations of drugs for the treatment of all infections due to *M. pyogenes*

even though certain in vitro studies have shown that combinations of antibiotics tend to delay the development of resistance in certain bacteria.[18] Reasons for this opinion are as follows: (1) most infections due to *M. pyogenes* will respond very well to the proper single antibiotic and, therefore, we do not feel justified in subjecting the patient to the dangers of more drugs than may be necessary for their cure; (2) since the increased incidence of strains of *M. pyogenes* that are resistant to antibiotics is due for the most part to an evolutionary type of change that is brought on by the selective pressure induced by the use of drugs, it is our feeling that there is a direct relationship between drug usage and the number of resistant strains. Therefore, routine use of drug combinations may ultimately increase the total number of resistant strains.[19] This argument, of course, does not hold for the use of combinations of drugs in the treatment of tuberculosis because in this disease, the organisms become resistant during therapy. This is in contradistinction to *M. pyogenes* where the resistant organisms usually are the initial pathogens. A controlled study in which routine combined therapy against *M. pyogenes* would tend to stabilize the sensitivities of the strains of *M. pyogenes* in an institution might, of course, refute these theoretical objections.

On the other hand, fulminating and certain other inaccessible infections undoubtedly should be treated with more than one agent. When possible these cases should be treated on the basis of sensitivity tests made with the organism isolated from the patient. However, the results reported may be of some aid when treatment must be begun before the results of the sensitivity tests are available. It should be emphasized that one of the distinct advantages in the use of the tube dilution test for determining bacterial sensitivity to antibiotics is the fact that combinations of drugs as well as single drugs may be tested for both bacteriostatic and bactericidal activity. The results of similar studies with the tube dilution method have proved applicable to the treatment of Proteus infections with penicillin and chloramphenicol, to the treatment of infections due to *Pseudomonas aeruginosa* with oxytetracycline and polymyxin B., and to the treatment of enterococcal endocarditis with chlortetracycline and erythromycin, so it is not unlikely that further studies will prove these results to be applicable for the treatment of certain infections due to *M. pyogenes*.[10-12]

No attempt was made in this study to delineate the mechanism by which inhibition, additive effects, or potentiation was obtained. However, it seems most likely that the advantageous effects were obtained by the fact that the drugs inhibit different metabolic reactions and that the inhibitory effects were obtained by interference with growth by one antibiotic so that the most advantageous part of the growth cycle for the action of the second agent was not reached.[20, 21]

Our results did not entirely agree with those of Manten[22] who found rather marked bactericidal inhibition when erythromycin and penicillin were combined. However, this discrepancy may be due to the fact that they used the replica plate technique which is quite different from the tube dilution method. In addition, studies in mice also failed to demonstrate inhibition due to the combination of erythromycin with penicillin.[23]

SUMMARY AND CONCLUSIONS

Each of various combinations of 12 antimicrobial agents have been tested in vitro for their bactericidal and bacteriostatic effectiveness against 10 strains of *M. pyogenes.* Additive, potentiating, and inhibitory results were found with almost every combination of drugs. No one combination or type of combination was markedly superior to the others. These results may have application in the selection of combinations of drugs either for use in in vitro studies of strains isolated from a specific infection or for use in a patient when time cannot be spared for in vitro studies.

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EPIDEMIOLOGIC STUDIES IN AN OUTBREAK OF HISTOPLASMOSIS

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A NUMBER of instances of focal outbreaks of histoplasmosis have been reported in recent years. One of the earliest and most adequately studied of these episodes occurred on a farm in Indiana.[1] In this outbreak, *Histoplasma capsulatum* was recovered from a patient as well as from the soil and the content of an unused silo. In many subsequent studies, much reliance has been placed upon circumstantial evidence, that is, isolation of *Histoplasma capsulatum* from the soil, dust or content of a silo to relate this organism to clinical disease. Grayston and Furcolow[2] reported a survey of these epidemics and the related epidemiologic data.

The small outbreak we have had an opportunity to study offered interesting avenues for investigation, the results of which appear worthy of publication.

CASE REPORTS

Two sisters, aged 2 and 4 years, respectively, entered the Children's Hospital, Cincinnati, Ohio, in December, 1952.

The younger patient, C. S., was admitted on December 16, because of loss of appetite of three weeks' duration, low-grade fever with an insidious onset, and finally a high fever for four days prior to admission. She exhibited enlarged cervical lymph nodes and enlargement of the liver and spleen. Râles and rhonchi were audible throughout both lung fields. She expired three days after admission. The diagnosis of disseminated histoplasmosis was established by blood and bone marrow cultures and was confirmed by autopsy examination.

The older sister, M. S., was admitted on December 17. She had painless enlargement of cervical lymph nodes and slight anorexia. There was also moderate hepatosplenomegaly. In this case, too, the diagnosis of histoplasmosis was established by cultures of bone marrow and lymph node tissue. Death occurred approximately seven months later. Autopsy was performed and revealed widespread histoplasmosis.

The details of the clinical, mycologic, and autopsy findings in both patients have been reported elsewhere.[3]

EPIDEMIOLOGIC STUDIES

Prior to March, 1952, the 2 patients, an older sibling, and their mother dwelt in the basement of a single story wooden frame building in Reading, Ohio. Five other adults and a 5-year-old child lived in the main portion of this house. In addition, there were 2 dogs and 2 cats maintained in the house. The children were not known to be ill while they lived in Reading.

In March, 1952, the children moved to a farm in Pisgah, Butler County, Ohio, approximately 8 miles from their Reading address. The farm was occupied by their maternal

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grandmother. Living there also were 3 maternal uncles (aged 6, 8, and 19 years), and 3 maternal aunts (aged 10, 14, and 16 years). In September, 1952, the children returned to the Reading home but continued to spend week ends at the farm.

The maternal grandmother and her family were housed in a reconditioned barn on the farm (Fig. 1). Cattle and pigs had previously occupied the barn but had been moved out several years earlier. A new floor had been laid and beaverboard partitions were installed to provide separate rooms.

Much debris, of the type which accumulates in city dumps, was scattered about the ground near the barn. Approximately 50 feet away from the barn was an old chicken coop which had been converted into a storehouse for miscellaneous articles. A space, approximately 6 by 6 feet, had been cleared in the center of the chicken coop in order that the children could use the structure as a playhouse. Two dogs, 3 cats, 2 ducks, and 3 chickens roamed freely over the grounds, but often sought shelter in the chicken coop. The dogs and cats had free access to the reconditioned barn and frequently slept with the children. Rats and mice were known to infest the area; no pigeons were kept.

Fig. 1.—This illustrates part of the farm. The larger structure on the left is the reconditioned barn. The smaller structure on the right is the chicken coop which the children used for a playhouse.

Close questioning of the various occupants of the farm failed to indicate any clear-cut illness recognizable with certainty as histoplasmosis. However, it was learned that the 6-year-old maternal uncle who resided in the barn had been hospitalized in October, 1951 with "atypical pneumonia." In addition, the paternal grandmother gave a history of "atypical pneumonia and pleurisy" which occurred in July and August, 1952, shortly after her first visit to the farm. She had daily febrile episodes, developed "anemia," and had "knots" over her body. Her illness lasted six to eight weeks; there had been no response to antibiotic therapy. She recovered spontaneously without residuum.

Histoplasmin skin sensitivity tests were carried out on various members of the family and contacts. A 1:1000 dilution of histoplasmin antigen was used in all cases. The patients' mother and their older sibling, who had remained well, exhibited positive reactions. In addition, 3 of the 5 adults and the 5-year-old child occupying the home in Reading were also found to be positive. Moreover, all the members of the family normally

resident at the farm, with the exception of the maternal grandmother, were positive reactors. The maternal grandmother was tested with 2 different histoplasmin antigens on 3 different occasions (until July, 1953). She failed to show any reaction (Fig. 2). The patients' father and the paternal grandmother, who resided at a different Reading address, were also positive reactors to histoplasmin.

Complement fixation tests were positive in high dilutions in the two patients (1:128 and 1:512) and in 1:2 dilution in their older sibling. All other individuals had negative complement-fixation tests.

The 2 dogs on the farm gave positive reactions to undiluted histoplasmin introduced intradermally. They were sacrificed. Both showed histoplasmosis on microscopic examination of sections of lungs, liver, spleen, and lymph nodes. Tissue cultures from the livers and spleens of both dogs were made. *Histoplasma capsulatum* was grown from only one animal, and was present in both liver and spleen.

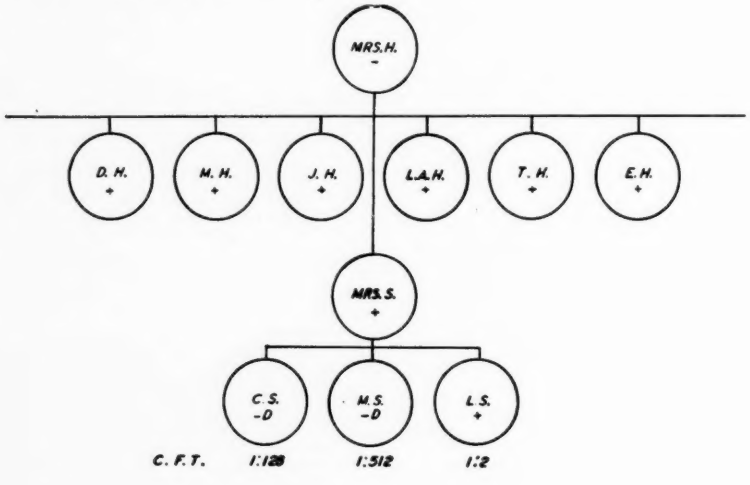

Fig. 2.—Representation of the patients' maternal family tree. Mrs. H. is the patients' maternal grandmother; Mrs. S., the patients' mother; L. S., the patients' elder sibling; and the remainder the patients' maternal aunts and uncles. Their corresponding skin test reactions are indicated.

A third dog was brought by us to the farm in February, 1953, in an attempt to produce experimental infection with *Histoplasma capsulatum*. This dog was healthy and negative to the histoplasmin skin test. In June, 1953, a positive skin test reaction was present. This dog, too, was sacrificed and was found to have histoplasmosis. Organisms were cultured freely from the tissues of this animal.

The 3 cats were negative to histoplasmin. One was sacrificed and autopsy showed no evidence of histoplasmosis.

Two chickens and a duck were sacrificed. All of these were found to have nonspecific hepatitis of minimal character. In one chicken, a single small granulomatous lesion was encountered in the liver. No organisms were identified in microscopic section or culture of this lesion.

Fourteen wild mice were trapped on the farm. None showed *Histoplasma capsulatum* infection.

Twenty-five soil* and water samples from the farm and 8 soil samples from the Reading address were injected into a total of 165 mice. Tissue cultures from the liver and spleen of these mice after four weeks were all negative for *Histoplasma capsulatum.*

GEOGRAPHIC AND GEOLOGIC SURVEY

Dr. Kenneth E. Caster, Professor of Geology at the University of Cincinnati, described the farm as follows:

> The farm is approximately one mile south of Pisgah, Butler County, Ohio (Mason Topographic Quadrangle, U. S. Geographical Survey). The elevation of the farm is about 880 feet A. T. (above tidewater). It lies in the headwaters of Sharon Creek, a tributary of the Mill Creek, which in turn flows into the Ohio River at Cincinnati. The farmhouse and garden are some 30 to 40 feet above the water in the small runnel of the Sharon Creek network. It is well drained.
>
> Pisgah, at 982 feet A. T., lies on the east-west line of the last terminal moraine of the Ice Age (Wisconsin glacial episode). The soil of the roadcut in front of the farm buildings and in the vegetable garden behind the barn which has been remodeled for human occupancy has the usual silvery sheen and silty texture of the Illinoisan residual glacial till.
>
> There is nothing peculiar about the geology or geomorphology of the site, except its position in the headwaters of the dissection of the Illinoisan glacial plain. The Sharon glacial lake appears never to have backed waters as high as the farm site in its upper headwaters, since the overflow col was at approximately 700 feet A. T.

DISCUSSION

The historical data gathered seemed to indicate that the farm occupied by the maternal grandmother represented the site of exposure to *Histoplasma capsulatum* in the two patients serving as the basis for this report. The positive reaction to histoplasmin skin test of all the members of the family normally dwelling at the farm, with the exception of the maternal grandmother, and the eventual demonstration of histoplasmosis in the two dogs there were considered supportive evidence. Reasonable proof that the site of exposure was the experimental production of disseminated histoplasmosis in a third dog which was originally healthy and negative to histoplasmin skin test when first brought to the farm.

Attempts to uncover the etiological organism by culture from the soil and scrapings of dust and water were unsuccessful. The technique used in this procedure had been successful previously. The method had served adequately in the isolation of *Histoplasma capsulatum* at the "Cincinnati Water Tower Epidemic."[2, 4] It is possible that the organisms could have been demonstrated with the examination of more soil samples. The search, however, was discontinued when the disease was produced experimentally in a third dog.

The negative response of the patients' maternal grandmother to the histoplasmin skin test is interesting. Our experience in a general hospital population had shown that individuals over the age of 50 exhibit a disproportionately low incidence of skin positivity, while the lower age group from the same area reveals positivity as high as 70 per cent.

*Including several from the chicken coop.

Geographic and geologic details have offered no additional salient information to this study. The occurrence of certain fungus diseases (histoplasmosis and coccidioidomycosis) predominantly in certain areas is a well-known fact. It is possible that geographic and geologic data may bring light in future epidemiologic studies.

SUMMARY

Two sisters, 2 and 4 years old, succumbed to disseminated histoplasmosis. The diagnosis was established by demonstration of *Histoplasma capsulatum* in cultures of bone marrow, blood, and lymph node tissue in both children. Autopsy examinations were confirmatory. Epidemiologic and immunologic studies indicated that a farm occupied by the maternal grandmother appeared to be the site of exposure. Further evidence was provided by the development of disseminated histoplasmosis in a dog originally free of the disease, after it had been maintained purposely in the suspected area.

Geographic and geologic details are appended.

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LABORATORY AND CLINICAL STUDIES OF D-GLUCURONOLACTONE ISONICOTINYL HYDRAZONE IN PULMONARY TUBERCULOSIS

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A GLUCURONOLACTONE derivative of isonicotinic acid hydrazide with high antituberculous activity, but considerably less toxicity than the parent compound, has been described[1, 2] and used in animal and human tuberculosis by a group of workers in France[3, 4] and another in this country.[5, 6] The French state that its low toxicity makes possible the administration of comparatively large amounts, that its antituberculous activity is at least equivalent to that of isoniazid and that some strains of tubercle bacilli and other organisms resistant to isoniazid fail to grow in similar or lower concentrations of this derivative. The biologic role of glucuronic acid in detoxication, reports of powerful synergism between glucuronolactone and streptomycin,[7, 8] recent evidence on reduction of toxicity of isoniazid from (a) Prescott and associates[9] by simultaneously administering glucuronate and glycine to mice and (b) Bond and Peoples[10] by the formation of hydrazones of isoniazid and uronic acids, suggests that this new compound merits careful consideration in the treatment of tuberculosis. In addition, De Moen Janssen, Van de Keere, and Zivkovic[11] have presented evidence that in human beings, the glucuronolactone derivative is much less susceptible to acetylation and metabolic breakdown in general than is INH. This article presents laboratory studies of comparative drug concentrations in plasma after the administration of this compound and isoniazid and clinical results in a group of eleven patients at the end of six months of treatment with d-glucuronolactone isonicotinyl hydrazone (INH-G, "Gatalone").

MATERIAL AND METHODS

Plasma Drug Concentrations.—These levels were studied in seventeen patients who received INH-G* and in eleven who received isoniazid; seven were given both drugs in approximately equivalent doses (in terms of INH†) at separate times with an intervening week of no chemotherapy. Plasma concentrations were determined before, and at intervals after, a single oral dose and a single day of three or four doses. In addition, plasma level curves were determined after a week or more of continuous treatment with INH-G in seven and isoniazid in five cases; two of these patients received both drugs. Single doses of INH-G (in terms of INH) ranged from 1.3 to 3.4 mg. per kilogram of body weight and those of INH from 1.4 to 3.7 mg. Total daily dosage of INH-G (in terms of INH) ranged from 4.0 to 11.8 mg. per kilogram of body weight and those of INH from 4.2 to 12.8 mg.

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†D-glucuronolactone isonicotinyl hydrazone owes 45.76 per cent of its weight to isonicotinic acid hydrazide.

Drug concentrations in oxalated fasting blood plasma were determined in duplicate immediately after withdrawal of blood samples by the method of Rubin and associates[12] for isonicotinic acid hydrazide with minor modifications.[13] This method is based on oxidation to isonicotinic acid, measured spectrophotometrically by the color reaction with cyanogen bromide in ammonia buffer. In our hands, recovery and conversion of both isoniazid and INH-G added to plasma in concentrations similar to those encountered after administration of these drugs was between 90 and 100 per cent. At and below 0.5 μg per milliliter, which we considered the lower limit of sensitivity of the method, recovery appeared to be somewhat greater than 100 per cent.

Selection of Cases for Clinical Study.—Eleven patients with pulmonary tuberculosis were selected for this pilot study. All were male veterans ranging from 27 to 57 years of age. Eight of the eleven were less than 45 years old. Six were white and five were Negro. None had received previous antituberculous therapy. In all cases the appearance of chest roentgenograms was consistent with a considerable exudative component likely to respond to effective chemotherapy. The disease was far advanced in nine cases and moderately advanced in two. It was bilateral in six cases and unilateral in five, and cavities were present in all cases. Diameters of the cavities were 1 cm. in two cases and ranged from 3 to 7 cm. in nine. Duration of symptoms before treatment was less than six months in eight cases, nine months in one, and one year or more in two.

Regimen.—All patients on treatment received 1,600 mg. INH-G* by mouth divided into four doses daily. Because of neurotoxic manifestations, the dosage was reduced to 1,200 mg. daily during the fifth month in one case (No. 3) and at the beginning of the third month in one (No. 11). Ten of the eleven cases completed eight months of therapy and one completed six months. None of the patients received collapse therapy during the period of treatment.

Bacteriology.—Sputa were examined before the drug was given, at approximately two-week intervals for the first two months of treatment and once a month thereafter except for Patient No. 11, whose organisms were studied at three and six months. Drug-containing media were prepared by adding to liquefied media (75 per cent nutrient agar and 25 per cent fresh egg yolk) aqueous solutions of either isonicotinic acid hydrazide or its glucuronolactone derivative which had been filtered through sintered glass. Drug concentrations in INH-G media were based on its content of INH. Trisodium phosphate concentrates of sputa were planted on both INH and INH-G media; drug concentrations of 0.2, 1.0, 5.0, and 10.0 μg per milliliter of medium were used. If acid-fast bacilli were too few for dependable direct testing in this manner, indirect tests were made with Dubos' medium subcultures of the entire growth on non-drug-containing medium. Final readings of direct tests were made after four weeks' incubation at 37° C. and of indirect tests after two weeks. The amount of growth was recorded according to the recommendations of the Committee on Evaluation of Laboratory Procedures of the American Trudeau Society.[14]

RESULTS

Plasma Concentrations of Drug.—Since the form in which INH occurs in the blood when INH-G is administered has not been established, all values in the present studies have been reported as μg of isonicotinic acid per milliliter of plasma.

Peak levels after a single dose of isoniazid occurred at 1 or 2 hours in all cases. After a single dose of INH-G, curves took one of two shapes, apparently depending largely on rapidity of hydrolysis or absorption of the drug. In eight of seventeen cases, the rapid rise and decline in plasma concentrations typical of isoniazid occurred, with peaks at 1 or 2 hours (Fig. 1, cases 3-IG and 5-IG; Fig. 2, cases 8-G, 10-G, 13-G, 14-G, 16-G, and 17-G). Of the remaining nine cases, one had a peak at 4 hours (Fig. 2, case 15-G) and in one, values were the

*10.8 to 13.9 mg. INH equivalent per kilogram of body weight.

same 4 and 6 hours after a single dose and 11 hours after the last dose of a full day of treatment (Fig. 1, case 7-IG). In the remaining 7 cases, a slow gradual rise occurred up to 4 or 6 hours after a single dose, and in five of the 7, a further rise was demonstrated 11 hours after the last dose of a full day of treatment (Fig. 1, cases 1-IG, 2-IG, and 4-IG, and Fig. 2, cases 9-G and 12-G), while in two cases, values were practically the same 6 hours after a single dose and 11 hours after the last dose of a single day's treatment (Fig. 1, case 6-IG, and Fig. 2, case 11-G).

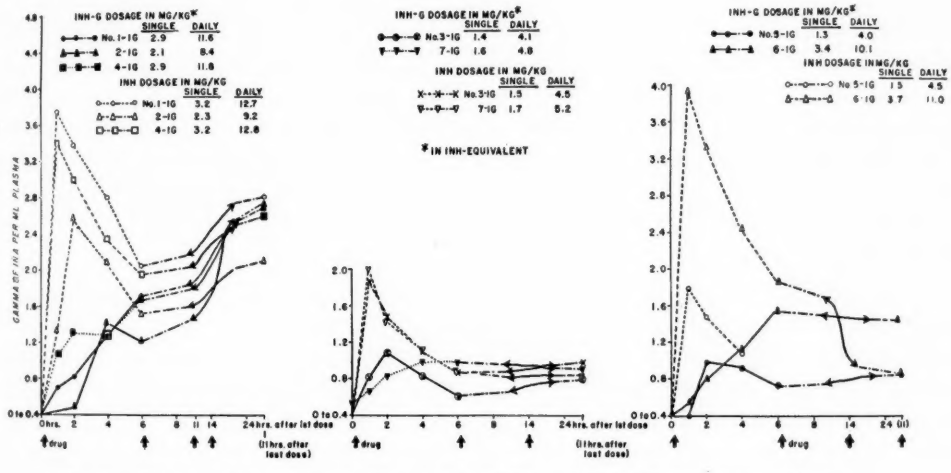

Fig. 1.—Plasma drug concentrations after INH and INH-G in same patients.

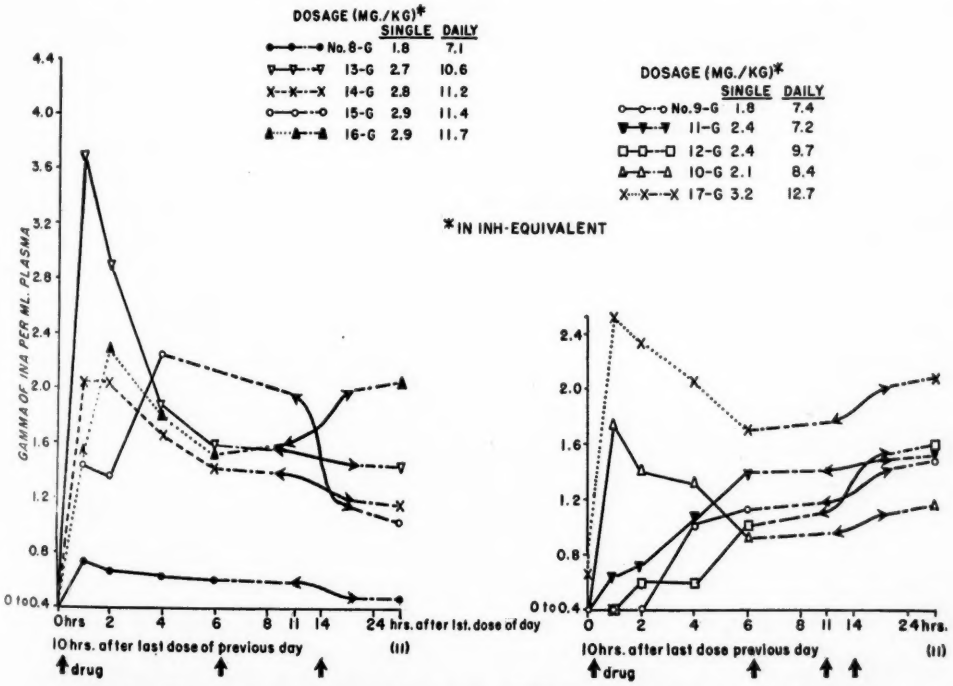

Fig. 2.—Plasma drug concentrations after INH-G Single dose and single day.

The shape of the curve appeared to be unrelated to the dosage. A rapid rise with peaks at 1 or 2 hours resulted from single doses varying from 1.3 to 3.2 mg. (in terms of isoniazid) per kilogram of body weight while 1.8 to 3.4 mg. (in terms of INH) produced the slow gradual rise.

In the seven cases given both drugs, levels during the first 6 hours after a single dose of the derivative were never as high as after equivalent doses of isoniazid (although they approached them in the 4- to 6-hour periods), but values 11 hours after the last dose of a single day of treatment with the two drugs compared very favorably, being actually somewhat higher after INH-G in two cases (2-IG and 6-IG, Fig. 1). Drug levels were not obtained later than 6 hours after a single dose of INH-G because of the inadvisability of fasting patients for so long a period. It is possible, therefore, that highest levels after a single dose of INH-G were not detected in the group exhibiting a gradual rise. Two patients of the group which received both drugs, however, had peak levels 2 hours after a single dose of INH-G with subsequent declines (Fig. 1, cases 3-IG and 5-IG); in both cases, values were somewhat lower after the derivative than after isoniazid per se.*

It was possible to produce rapid peak drug levels with INH-G which were as high in some cases as those resulting from isoniazid in others. Of the eight INH-G cases with typical isoniazid curves, peak levels of five were as great as the highest of those on isoniazid; in one case, the derivative had been administered in a smaller dose (Table I, case 13-G, compared with cases 1-IG, 4-IG, and 6-IG); in the rest the reverse is true. The remaining three INH-G patients in this category (i.e., with typical isoniazid curves) constitute a group in which peak plasma concentrations were considerably lower than the lowest of the isoniazid cases (Table I, cases 8-G, 3-IG, and 5-IG, compared with cases 3-IG,

TABLE I. HIGHEST RAPID PEAK DRUG CONCENTRATIONS IN PLASMA AFTER SINGLE DOSES OF INH-G AND INH
AND
PEAK DRUG CONCENTRATIONS AFTER THE USUAL SINGLE THERAPEUTIC DOSE OF ISONIAZID (100 MG.) GIVEN AS INH AND INH-G

DRUG GIVEN:	INH-G		INH		
PEAK CONC. μG. INA/ML.	DOSE* MG./KG.	CASE NO.	PEAK CONC. μG. INA/ML.	DOSE MG./KG.	CASE NO.
3.69 (1 hr.)	2.7	13-G	3.74 (1 hr.)	3.2	1-IG
			3.50 (1 hr.)	3.2	4-IG
			3.94 (1 hr.)	3.7	6-IG
2.52 (1 hr.)	3.2	17-G	2.57 (2 hr.)	2.3	2-IG
2.27 (2 hr.)	2.9	16-G	2.14 (1 hr.)	1.5	10-I
2.05 (1, 2 hr.)	2.8	14-G	1.98 (1 hr.)	1.8	7-IG
1.74 (1 hr.)	2.1	10-G	1.87 (1 hr.)	1.5	3-IG
0.74 (1 hr.)	1.8	8-G	2.42 (2 hr.)	1.9	11-I
1.09 (2 hr.)	1.4	3-IG	1.87 (1 hr.)	1.5	3-IG
0.99 (2 hr.)	1.3	5-IG	1.78 (1 hr.)	1.5	5-IG
			1.20 (2 hr.)	1.4	8-I
			1.82 (1 hr.)	1.5	9-I

*Dosage based on INH-content.

*Highest concentrations after INH-G were 1.09 and 0.99 μg per milliliter, respectively, both at two hours; after isoniazid they were 1.87 and 1.79 μg per milliliter, respectively, both at one hour. Dosage in both cases had been 1.3 to 1.5 mg. (in terms of INH) per kilogram of body weight.

5-IG, 7-IG, 8-I, 9-I, 10-I, and 11-I). Dosage in all patients of the latter group had been similar to the usual single therapeutic dose of 100 mg. of isoniazid, i.e., 1.3 to 1.9 mg. (in terms of isoniazid) per kilogram of body weight.

After a week or more of continuous treatment, curves were similar to those obtained during the first day except that with INH-G, high levels occurred earlier after the first dose of the day (Fig. 3, cases 6-IG and 7-IG, compared with the same cases in Fig. 1) and appeared to be somewhat more constant than after the parent compound.

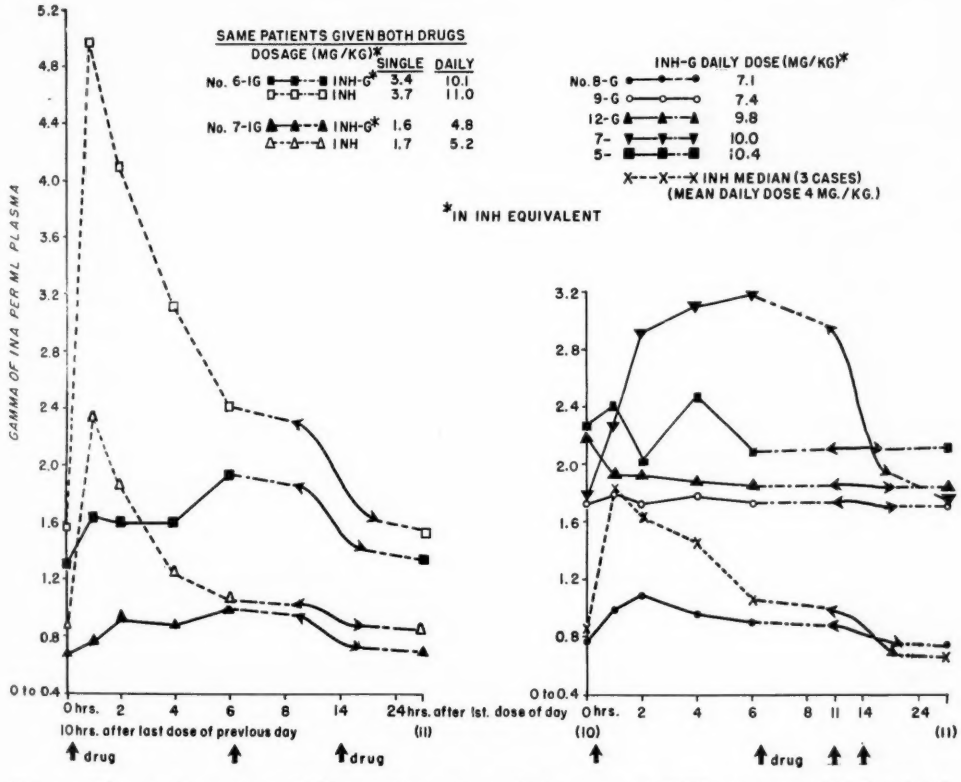

Fig. 3.—Plasma drug concentrations after one week or more of INH given as (A) isoniazid and (B) glucuronolactone.

Clinical Results.—General condition improved in the eleven patients treated with INH-G although symptomatic relapse occurred during the fifth (No. 7) and sixth (No. 8) months in two. Maximum temperatures ranged from 100° to 103° F. in nine patients at the start of treatment and subsided to normal in all (usually within two months); however, fever recurred in two cases (No. 7 and No. 8). Two patients were afebrile at the start of treatment and remained so. Cough and expectoration disappeared completely in three cases and substantially lessened in seven, but increased again by the fifth month in two (No. 7 and No. 8). One patient had no cough or expectoration before treatment. All patients gained weight ranging from 5 to 38 pounds (average 23.6 pounds).

TABLE II. THE EFFECT OF 6 MONTHS' TREATMENT OF PULMONARY TUBERCULOSIS WITH 1,600 MG. DAILY OF D-GLUCURONOLACTONE ISONICOTINYL HYDRAZONE (10.8 TO 13.9 MG. INH EQUIVALENT/KG. BODY WEIGHT)

CASE NO.	DISEASE AT START			AFTER 6 MOS. INH-G			DRUG SUSCEPTIBILITY OF TUBERCLE BACILLI						
	STAGE	DURATION	CAVITY	TEMP. DROP	WT. GAIN	X-RAY IMPROV.	PRE-R	1 MO.	2 MO.	3 MO.	4 MO.	5 MO.	6 MO.
1	Far adv.	Old	6 cm.	Norm. 2 mo.	24.0	Moderate	S*	I*	R*	R	R	R	R
2	Mod. adv.	Recent	1 cm.	Norm. from start	19.5	Marked	S	O	O	O	O	O	O
3	Far adv.	Recent	1 cm.	Norm. 3 wk.	17.5	Marked	S	O	O	O	+O	S	+O
4	Far adv.	Old	7 cm.	Norm. 3 wk.	38.0	Moderate	S	S	S	S	O	S	S
5	Far adv.	Recent	3 cm.	Norm. 2 mo.	36.5	Slight	S	S	-	-	-	O	O
6	Far adv.	Recent	6 cm.	Norm. 2 mo.	12.0	Slight	S	S	S	°N.V.	I	I	I
7	Far adv.	Recent	3 cm.	Rose after drop	32.0	Worse	S	S	O	R	S	R	R
8	Far adv.	Recent	3 cm.	Rose after drop	5.0	Worse	S	S	R	R	R	R	R
9	Far adv.	Recent	4 cm.	Norm. 4 mo.	27.0	Moderate	S	S	O	-	R	O	I
10	Mod. adv.	Recent	3 cm.	Norm. from start	24.0	Slight	S	S	O	O	O	+O	O
11	Far adv.	9 mo.	5 cm.	Norm. 1 mo.	25+	Marked	-	-	-	R	-	-	R

*S = 0 to 20 colonies on drug-containing medium.
R = 75 per cent to 100 per cent of control growth on 1 μg or more per milliliter.
I = from 21 colonies to 75 per cent of control growth on 1 μg or more per milliliter.
- = No test.
0 = No acid-fast bacilli by smear or culture.
+0 = Positive smear, negative culture.
°N.V. = Negative smear, 5 colonies on cultures, no growth in subcultures.

Roentgenological.—At the end of six months of treatment, x-rays revealed marked improvement in three cases, moderate improvement in three, slight improvement in three, and two cases (No. 7 and No. 8) were worse at the fifth and sixth months, respectively (Table II). The two cases with longest known duration of disease before treatment were both far advanced and both showed moderate roentgenographic improvement, whereas roentgenographic worsening was noted in two far-advanced cases of recent onset. The cavities were lost to view in two cases (both 1.0 cm. cavities), became smaller in three, remained unchanged in four, became larger in one (No. 7) and in the remaining case (No. 5), the open cavity became filled and was represented roentgenographically by a round sharply circumscribed homogeneous density.

Bacteriology.—The time of development of resistance to the two drugs was parallel and tubercle bacilli resistant to isoniazid were equally resistant to INH-G on the basis of the isonicotinic acid hydrazide contained in the medium.

Six months after the start of treatment, cultures of seven of the eleven cases were still positive for tubercle bacilli (63.6 per cent). Cultures from four (36.3 per cent) were negative at this time, but conversion of one of these cases (No. 3) was questionable because acid-fast bacilli had been seen in smears of six-month specimens, and both smears and cultures had been positive at five months (Table II). Bacilli from four of the eleven cases (36.3 per cent) were resistant to both INH and INH-G; in one case they were still susceptible (9 per cent) and in two, of intermediate resistance (18 per cent) (see legend, Table II, for definitions of terms). On the basis of the number of patients still discharging culturable tubercle bacilli after six months of treatment, 57.1 per cent were resistant, 14.3 per cent were susceptible, and 28.5 per cent were of intermediate resistance.

DISCUSSION

Results of the present study appear to be somewhat at variance with those of certain other investigators of this compound, who make the following claims: (a) equal effectiveness with isoniazid in animals on a weight to weight basis,[3, 4, 6] (b) no detectably resistant bacilli after eight months of administration,[6] and (c) susceptibility of certain strains of isoniazid-resistant bacilli.[4]

In vitro studies* have shown that at 37° C. a large amount of INH-G in solution in distilled water, plasma, serum, and borate buffer is converted to free isonicotinic acid hydrazide during the first hour (35 per cent in water of pH 5.0 to 5.6; 50 to 70 per cent in plasma of pH 8.2 or serum of pH 8.2; 100 per cent in borate buffer of pH 8.2).[26] Hydrolysis in water is complete after 24 hours, but longer incubation in plasma or serum (24 hours) caused little change in the percentage of the free hydrazide. In veronal buffer, hydrolysis was slower and little affected by a drop in hydrogen-ion concentration from pH 7.35 to 8.1. However, an INH-G concentration sufficient to change the pH of veronal buffer from 7.35 to 6.9 in 24 hours and 6.8 in 72 hours resulted in an increase

*Using a method for distinguishing between INH and INH-G developed by Dr. S. A. Peoples[25] of the University of California, Davis, California.

in hydrolysis from 32 per cent in 24 hours to 46 per cent in 72 hours, whereas a concentration so low that it did not affect the pH significantly hydrolyzed little, if any, beyond the 24-hour period.[26]

The shape of curves of plasma concentrations after administration of INH-G suggests that the rapidity of the appearance of high drug concentrations in the blood is a highly individual matter. Hydrolysis of the glucuronolactone isonicotinyl hydrazone molecule is probably affected by both hydrogen-ion concentration and enzyme activity in the gastrointestinal tract. Rapidity of hydrolysis appears to be a more important factor in blood levels attained than speed of absorption, for isoniazid administration caused a rapid rise in blood levels in the same patients exhibiting slow gradual rises after INH-G. The fact that rapid appearance of high blood levels usually required higher doses of INH if given as INH-G, and lower peak concentrations with comparatively low doses, suggest that some of the derivative may be excreted without absorption.

An unsuccessful attempt was made to demonstrate INH-G in the blood stream of one patient after the ingestion of several doses of 600 mg. each (a daily total of 17 mg. INH equivalent per kilogram of body weight). In view of rapid in vitro hydrolysis, negative evidence (even though analyses were made immediately) does not constitute proof that the glucuronolactone compound fails to enter the blood stream. Failure to demonstrate even small quantities of INH-G, however, together with evidence of rapid in vitro hydrolysis, suggests that large portions of INH-G appear in the blood in the form of the free hydrazide. The question of its value as a chemotherapeutic agent therefore resolves itself into the following: (a) whether or not high doses of isoniazid are more effective in the treatment of tuberculosis than the usual well-tolerated therapeutic amount and (b) whether or not higher doses are better tolerated if administered as the glucuronolactone.

In the present series of treated patients, symptomatic response was very favorable and roentgenologic resolution of infiltration was satisfactory at the end of six months of treatment with INH-G, whereas the incidence of cavity closure and sputum conversion were not remarkable. Substantial (moderate and marked) roentgenographic improvement was noted in six of eleven patients (54.5 per cent) by the end of six months. Within the limits imposed by a small number of patients, these results compare reasonably well with those obtained in cases treated with 150 and 300 mg. of isoniazid daily for periods of five to eight months (38 per cent),[16] twenty-eight weeks (50.7 per cent),[17] and twenty-eight weeks (43.1 per cent).[18] Mount and his associates[17, 18] (U.S.P.H.S. cooperative study) found no statistically significant difference in the percentage of patients whose roentgenograms showed substantial improvement whether the patients were treated with isoniazid alone, or in combination with 12.0 Gm. of PAS daily or 1.0 Gm. of streptomycin twice weekly. Mount and Ferebee also reported that there was no statistically significant difference in the incidence of substantial roentgenographic improvement at twenty weeks[15] or at thirty-two weeks[19] between patients receiving 3 mg. of isoniazid per kilo-

gram of body weight daily and those receiving 10 mg. per kilogram (when isoniazid was given in combination with 12 Gm. of PAS daily or 1.0 Gm. of streptomycin twice weekly, or when the higher dose of isoniazid was given concurrently with both streptomycin and PAS). Thus there is no indication in the above reports or in results in our small series of cases that higher doses of isoniazid are actually more effective or that the glucuronolactone derivative has chemotherapeutic virtue aside from its content of isoniazid.

The incidence of peripheral neuropathy among patients receiving 1,600 mg. of INH-G daily (containing 732 mg. of isoniazid), which was 10.8 to 13.9 mg. of isoniazid-equivalent per kilogram of body weight, was 54.5 per cent by the third month and 63.6 per cent by the end of the fourth month of treatment. Other authors have reported peripheral neuropathy in 3.4 per cent[15] of patients who received 10 mg. of isoniazid per kilogram of body weight daily for twelve weeks or longer, 0.0 per cent[20] among thirty-two patients who received 5 mg. per kilogram of body weight daily for one year, and 8, 18 and 44 per cent of patients on daily dosages of 6 to 10 mg., 11 to 15 mg., and 6 to 24 mg. per kilogram daily, respectively.[21] Thus the incidence of peripheral neuropathy among our patients treated with INH-G was at least as high or indeed higher than the reported incidence among patients treated with equivalent or even higher doses of isoniazid per se. Whereas this could be an accidental finding in such a small group of patients, it was none the less surprising.

Development of bacillary resistance in this small series of cases was not unlike that occurring in patients treated with isoniazid alone.[16, 22, 23] In vitro studies indicated that resistance to the two drugs developed at about the same time, and to an equal degree, if concentrations in the media were calculated on the basis of the hydrazide, supporting Koch-Weser's contention that bacilli resistant to isoniazid are also resistant to the glucuronolactone derivative,[24] i.e., that it is merely resistance to isoniazid in both cases. Actually it is doubtful whether the effect of the intact glucuronolactone derivative on the growth of tubercle bacilli has ever been tested in vitro in view of laboratory evidence of rapid conversion of INH-G in solution at 37° C. to free hydrazide.[25, 26]

Koch-Weser's experiments with labeled compounds[24] indicate that INH-G affects the tubercle bacillus only through the isonicotinic acid hydrazide contained in and released from the molecule. The work in the present report supports his views and is consistent with the hypothesis that d-glucuronolactone isonicotinyl hydrazone acts as a reservoir of isonicotinic acid hydrazide, releasing it slowly to maintain a relatively constant level of the drug in the blood stream.

SUMMARY AND CONCLUSIONS

1. The oral administration of the glucuronolactone derivative of isonicotinic acid hydrazide to patients with pulmonary tuberculosis resulted in plasma drug level curves of two types: (a) a rapid rise and decline similar to that obtained after isoniazid, or (b) a slow gradual rise with a peak at 6 hours or later.

2. Isoniazid will produce a rapid rise in drug concentration in the same patients who respond to INH-G with a slow gradual rise, suggesting that rapidity of hydrolysis to INH in individual patients, or slower absorption of the larger molecule, may be factors in the type of curve obtained.

3. Drug levels up to 4 hours after a single dose of INH-G are lower than those in the same patients after a similar single dose of isoniazid; in 6 hours, they approach them and 11 hours after the third or fourth dose of a single day of treatment, plasma drug levels are similar with the two drugs.

4. Large doses of INH-G may result in plasma concentrations as high in certain cases as those produced by isoniazid in others; usually, but not always, more INH-G than INH is required. If the two drugs are administered in doses equivalent to the usual single therapeutic dose of isoniazid (100 mg. or approximately 1 to 2 mg. per kilogram of body weight), higher plasma concentrations occur with isoniazid.

5. Six months of treatment of 11 cases of pulmonary tuberculosis with INH-G (initially 1,600 mg., equivalent to 732 mg. of isoniazid, reduced to 1,200 mg. daily in two cases at the third and fifth months, respectively) resulted in clinical and roentgenological improvement comparable to that obtained by others with 300 mg. of isoniazid.

6. Neurotoxic symptoms in this small group were more frequent with 1,600 mg. of the glucuronolactone derivative daily (10.8 to 13.6 mg. of isoniazid equivalent per kilogram of body weight) than other investigators report with equal or higher doses (up to 24 mg. per kilogram) of isoniazid daily. Mild symptoms were controlled by the administration of vitamin B_6. There was no evidence of toxicity attributable to the renal, hepatic, or hematopoietic systems.

7. Sputum conversion and development of bacillary resistance were not unlike those reported by others after treatment with isoniazid. Resistance to the two compounds developed at about the same time, and bacilli resistant to one compound were equally resistant to the other in terms of the hydrazide.

8. There was no indication in this small series of cases that the higher dose of isonicotinic acid hydrazide in the form of the glucuronolactone derivative was more effective in the therapy of tuberculosis nor that the glucuronolactone had chemotherapeutic virtue aside from its content of isonicotinic acid hydrazide.

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TETRAZOLIUM REDUCTION BY TUBERCLE BACILLI

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THE tetrazolium salts and especially 2,3,5-triphenyltetrazolium chloride (TTC) have been described as indicators for oxidation-reduction enzymes.[1-3] At pH 7.0 the redox potential of TTC is 0.08 volt.[4] Under the influence of several dehydrogenase systems, and probably with simultaneous oxidation of DPN,[5] the water-soluble colorless compound triphenyltetrazolium chloride is reduced to the corresponding formazan, a red water-insoluble compound[6, 7] (Fig. 1). The method, originally used to indicate the viability of germinating seeds,[8, 9] has been employed widely to detect the oxidative activity of molds,[10] yeast,[11] and bacteria.[12-15] In a variety of normal, diseased, and tumor tissues either the "endogenous" TTC reduction without the addition of substrate[16-19] or the TTC reduction due to succinodehydrogenase after the addition of succinate[20, 21] was evaluated. The TTC-reducing ability of different elements in blood[22] and of fibroblasts in tissue cultures[23] was investigated.

The reduction of TTC and of neotetrazolium by tubercle bacilli and the resulting stain of the organisms by the formazan has been used to identify the polar bodies as mitochondria[24, 25] and to facilitate an earlier detection of growing tubercle bacilli.[26] It has also been suggested that TTC reduction might be a useful test for viability of tubercle bacilli.[27] In a preliminary communication[28] we reported on a quantitative method employing TTC reduction as an indicator for either presence or absence of metabolic activity of tubercle bacilli under various conditions. The experiments described here were designed to investigate further the value of TTC reduction as a measure of the metabolism of virulent human tubercle bacilli.

MATERIAL AND METHODS

Virulent human tubercle bacilli, strain H37Rv, were used throughout the experiments. Of these, 0.1 mg. wet weight were inoculated into test tubes standardized for the Coleman Nephelometer, containing 10 ml. of Dubos' liquid Tween* albumin medium.[29] The initial suspension of tubercle bacilli contained, therefore, a little more than 3 nephelometer units (N.U.) per milliliter.[30] The growth of the tubercle bacilli was followed by repeated nephelometer readings[31] and the amount of the bacilli in the suspension expressed in N.U. per milliliter. TTC was used as a 0.2 per cent solution in a phosphate buffer with a pH of 7.4, and 0.5 ml. of this solution was added to the 10 ml. suspension. After 24 hours (and in a few instances 8 hours) 0.5 ml. of 10 per cent Formalin was added to each tube. Formalin was found to inhibit the further reduction of TTC and did not change the color of the formazan. After 30 minutes 10 ml. of ethyl acetate was added, and the tubes were agitated repeatedly until

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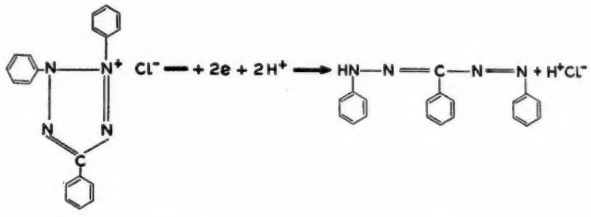
*Proprietary name of the polyoxyethylene derivative of Sorbitan monooleate, obtained from Atlas Powder Co., Wilmington, Del.

the formazan had been completely extracted by the ethyl acetate. When kept in the dark, the formazan in ethyl acetate does not change color in months. However, exposed to light for several hours the red color changes somewhat to yellow. The ethyl acetate phase was then allowed to separate on top of the aqueous phase, and after removal the concentration of red formazan was determined in the Lumetron colorimeter at 490 mμ. Previously, a standard curve had been prepared by totally reducing known concentrations of TTC with sodium hyposulfite in liquid Dubos medium and extracting and measuring the formazan as described above. The TTC reduction was expressed in micromoles per milliliter of suspension.

In 148 tubes growth was followed by nephelometer readings, and at intervals the average TTC reduction in 8 or 24 hours in at least 5 tubes was determined to verify the TTC reduction in different phases of growth.

To determine if tubercle bacilli have to be present for the reduction of TTC or if enzymes and/or other substances are secreted into the medium which suffice to reduce TTC, 20 tubes were used in which growth had reached 500 to 550 N.U. per milliliter. Bacilli and medium were separated by centrifugation and TTC reduction by the medium and by the bacilli suspended in fresh medium were measured.



Triphenyltetrazolium chloride Formazan

Fig. 1.—Reduction of Triphenyltetrazolium chloride to the corresponding formazan.

The influence of exposure of tubercle bacilli to different temperatures was investigated on 8 groups of 8 or 9 tubes each in which growth had reached an average of 450 N.U. per milliliter. One group was kept at 4° for sixteen hours before and while the TTC reduction was determined. Each of the other 7 groups was exposed for 30 minutes to a different temperature, ranging from 37° to 100° and immediately afterward the TTC reduction was measured at 37°.

To investigate further the influence of low temperature, growth and TTC reduction at 37° were measured in 120 tubes kept at 37° for 10 days, transferred for 10 days to 4° and finally returned to 37° for another 24 days.

The action of antituberculous drugs was observed in 175 tubes. To 86 tubes 10 μg of streptomycin (SM) per milliliter of medium and to 89 tubes 2 μg of isoniazid (INH) were added 6 days after inoculation, and growth and TTC reduction were followed.

RESULTS

It might be anticipated that TTC reduction would correlate with growth of tubercle bacilli. Superficially this would appear to be true, for in Fig. 2, as growth increases, there is an increase in TTC reduction after a short lag phase. Closer inspection of this graph indicates, however, that there continues to be TTC reduction even after growth has stopped and there seems to be a close relationship between the number of tubercle bacilli present in the medium and TTC reduction. To test this hypothesis further nephelometric readings, and TTC reductions of all the individual tubes represented in Fig. 2, regardless of age of culture or phase of growth, are correlated in Fig. 3. It is evident that the number of tubercle bacilli present, as expressed in nephelometric units (N.U.), and

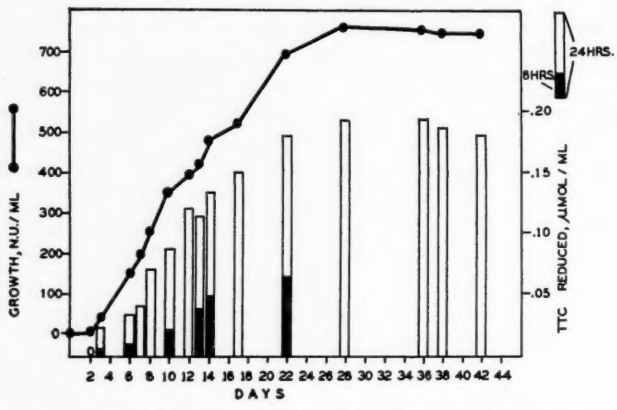


Fig. 2.—TTC reduction correlated with the growth of tubercle bacilli at 37°. The reduction in 8 hours was measured only at 3, 6, 10, 13, 14, and 22 days.

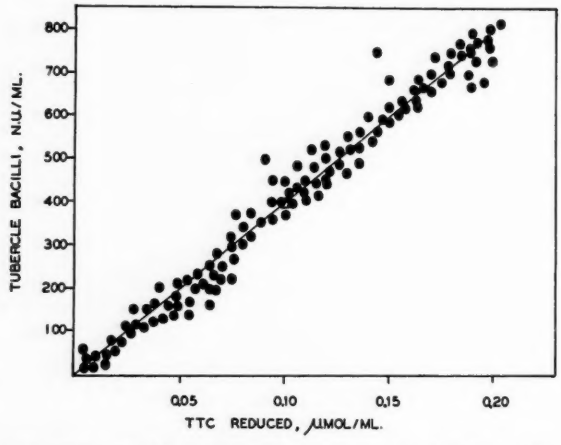


Fig. 3.—Correlation of TTC reduction with the number of tubercle bacilli given in nephelometer units (N.U.).

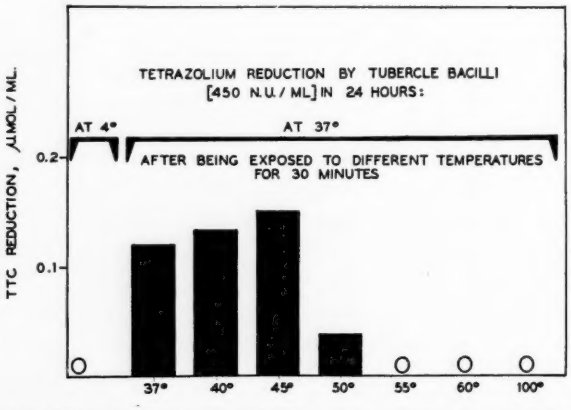

TETRAZOLIUM REDUCTION BY TUBERCLE BACILLI
[450 N.U./ML] IN 24 HOURS:
AT 4°
AT 37°
AFTER BEING EXPOSED TO DIFFERENT TEMPERATURES FOR 30 MINUTES
TTC REDUCTION, µMOL/ML.
37° 40° 45° 50° 55° 60° 100°

Fig. 4.—TTC reduction by tubercle bacilli. One group was kept at 4° before and while the TTC reduction was determined. The other groups were exposed to different temperatures before and to 37° while the TTC reduction was determined.

TTC reduction correlate well throughout. In other words the amount of TTC reduced depends only on the number of organisms in the medium and is not related to the phase of growth nor the age of the medium.

The medium decanted from growing tubercle bacilli did not reduce any TTC, while the tubercle bacilli in fresh medium reduced it in significant quantity.

Fig. 4 shows that bacilli which had been submitted to 55° or more for 30 minutes do not reduce TTC. Previous exposure to 50° decreases markedly the TTC reduction, while up to 45° no change or perhaps a slight enhancement occurs. Refrigerated bacilli do not reduce TTC. This was confirmed in the experiment represented in Fig. 5. Tubercle bacilli were grown at 37° for 10 days and then transferred to 4° for 10 days. Growth and TTC reduction ceased after the transfer to the refrigerator. When returned to 37° growth and TTC reduction were resumed after a lag phase of approximately 8 days.

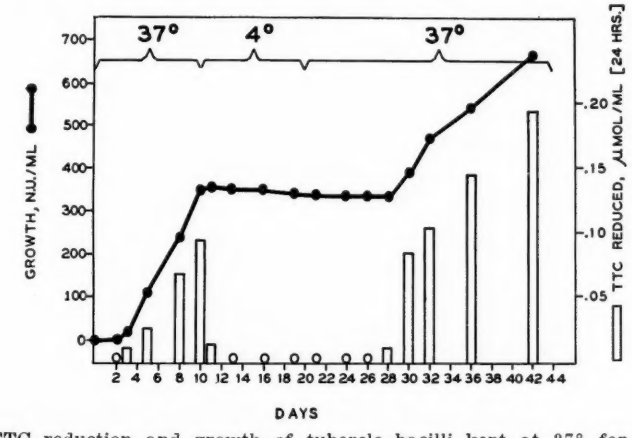


Fig. 5.—TTC reduction and growth of tubercle bacilli kept at 37° for 10 days, transferred to 4° for 10 days, and finally returned to 37° for 24 days. Twenty-four hour TTC reduction was measured at 37°.

After the addition of streptomycin (Fig. 6) TTC reduction, though markedly decreased, continues for about 3 days, while growth apparently stops the day after the drug is added. From the fourth day on with progressive lysis of the tubercle bacilli no TTC is reduced. In contrast, after the addition of INH, growth continues for about 6 more days and during this period a slowly decreasing TTC reduction is observed.

COMMENT

The data presented indicate the value of the TTC reduction test for the investigation of certain aspects of tubercle bacilli metabolism. As demonstrated in Fig. 2 and Fig. 3, TTC reduction depends on the number of tubercle bacilli. Under the present experimental conditions it does not seem to make a difference if these bacilli are growing or have ceased to grow, and if they are in the arithmetic or logarithmic phase of growth. Since Halpern and Kirchheimer[32] have shown that the change from logarithmic to arithmetic growth depends on lack of oxygen, and since the cessation of growth in liquid medium

depends on the same, one can conclude that TTC reduction under these experimental conditions does not depend on oxygen supply to the same degree as multiplication does. It has been shown previously that *E. coli*[13] as well as tissue slices from different organs[33] reduce more tetrazolium anaerobically than aerobically.

The fact that TTC is not reduced by cell-free medium in which tubercle bacilli had been growing indicates that either the enzyme system responsible for TTC reduction is not secreted into the medium or, in addition to the enzyme system in the medium, cell constituents have to be present. It was demonstrated that intact cells reduce more TTC than cell homogenates,[6] and that yeast dialysates do not reduce TTC until undialysed yeast is added, in which any enzymes possibly existing are destroyed by boiling.[11] Further experiments to clarify this for tubercle bacilli in liquid medium are under way.

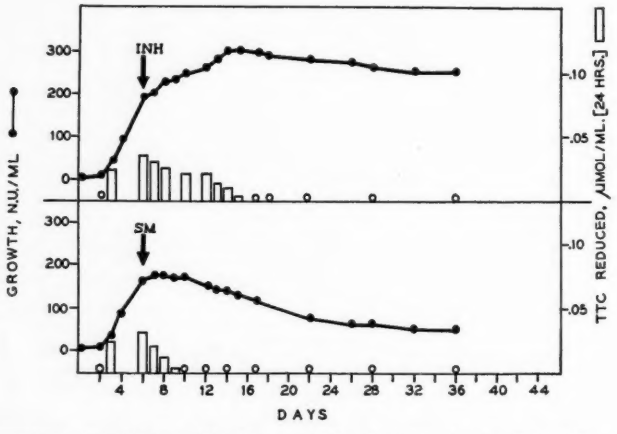


Fig. 6.—TTC reduction correlated with the growth of tubercle bacilli under the influence of isoniazid and streptomycin.

Exposure of tubercle bacilli to temperatures up to 45° (Fig. 4) does not decrease their ability to subsequently reduce TTC, while previous exposure to 55° to 100° inhibits the reaction completely. Complete inhibition also occurs at 4°. From Fig. 5, however, it becomes obvious that this lack of TTC reduction cannot be taken as a sign of loss of viability, since these bacilli, returned to their normal temperature, after a lag phase again take up the interrupted growth and reduce TTC. We described[34] a similar phenomenon in tubercle bacilli which, after being inhibited by streptomycin, ceased to grow and to reduce TTC. After being transferred into fresh medium and freed from the influence of streptomycin, following a long lag period they started to multiply and to reduce TTC again. The fact that these bacilli temporarily not reducing TTC under the influence of low temperature or of an antituberculous drug can resume their capacity to do so, demonstrates that TTC reduction cannot be taken as a viability test, but only as an indication for a certain metabolic activity.

Another demonstration that these "inactive" bacilli do not behave like "killed" or nonviable organisms is given by studies on the uptake of C^{14}-labeled

INH.[35] While formalin or heat-killed bacilli do not take up C^{14}INH, cold or streptomycin-inhibited bacilli take up even more C^{14}INH than untreated bacilli at 37°.

The comparison of growth curves and TTC reductions after the addition of antituberculous drugs (Fig. 6) shows clearly the difference of action of streptomycin (SM) and isoniazid (INH). The continued growth after the addition of INH[36] is paralleled by continuing, though decreasing, TTC reduction. This decreasing TTC reduction and growth parallels the decrease of acid-fast and increase of nonacid-fast organisms.[34]

After addition of SM, growth and TTC reduction cease rapidly, while acid-fastness remains.[34] The antituberculous drugs inhibit simultaneously growth and TTC reduction. But the rapidity of inhibition and the preservation of acid-fastness after SM indicate that its mechanism of antibacterial action must be quite different from that of INH.

SUMMARY

Triphenyltetrazolium chloride (TTC) is reduced by virulent human tubercle bacilli, strain H37Rv. The amount of TTC reduced correlates well with the number of tubercle bacilli regardless of phase of growth or age of medium. TTC is not reduced when the metabolism of the bacilli is temporarily or permanently inhibited by either low temperatures or antituberculous drugs. Removed, however, from the unfavorable conditions, the bacilli can resume growth and TTC reduction. TTC reduction proves, therewith, to be a valuable quantitative test for bacterial metabolism, but is not a test for bacterial viability.

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AUTOMATIC ROTATING CUFF APPARATUS

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THE clinical and physiologic value of venous tourniquets in acute heart failure associated with pulmonary edema has been amply documented. Constricting cuffs, bands, or tubing are usually applied to the proximal parts of the four extremities impeding the venous return to the heart. Unfortunately arterial occlusion has occasionally been produced, resulting in a loss of a limb. Another hazard of venous tourniquets has been the development of deep vein thrombosis, a result of uncontrolled venous stasis.

The purpose of this report is to present an automatic rotating tourniquet-cuff apparatus which permits precise controlled venous compression and minimizes the occurrence of the complication of venous thrombosis.

DESCRIPTION OF APPARATUS

The apparatus consists of a 110 volt AC-DC 1/70 H.P. motor, an air compressor, a bleeder valve, an air reservoir, a rotary air valve, an anaeroid manometer, four blood pressure cuffs, and a metal housing unit (Figs. 1 and 2).

The height of the pressure in the cuffs is controlled by the bleeder valve. The distribution and rotation of pressure in the cuffs is controlled by the rotary valve (*E*). A forty-five-minute clock motor (*F*) drives the rotary air valve (*E*) so that a complete rotation occurs in forty-five minutes. Every eleven and one-quarter minutes one cuff is completely deflated, and the other three cuffs are inflated to the pressure level determined by the control of the bleeder valve. Thus at the onset of operation of the apparatus, three cuffs are inflated to a desired level and the fourth cuff remains deflated. After eleven and one-quarter minutes, the previously deflated cuff is inflated, and one of the previously inflated cuffs is completely deflated. During this switching or rotation the other two cuffs are temporarily partially deflated to about one-half of their previous levels (Fig. 3). This feature of temporary partial deflation during switching was incorporated in the apparatus to prevent excessive venous stasis by permitting periodic temporary systemic venous decompression. The magnitude of the drop of pressure and the duration of partial deflation during switching depends on the cuff pressure before switching (Table I).

TABLE I

CUFF PRESSURE BEFORE SWITCHING (MM. HG)	LOWEST CUFF PRESSURE DURING SWITCHING (MM. HG)	DURATION PARTIAL DEFLATION (SECONDS)
30	15	15
40	22	15-17
60	36	21
80	50	21

The cuff on each extremity is thus automatically completely deflated for eleven and one-quarter of each forty-five minutes, and is fully inflated for the remainder of the time except for two fifteen-to-twenty-second periods of partial deflation at the time of rotation or switching (Fig. 3).

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**From the Rand Development Corporation, Cleveland, Ohio.

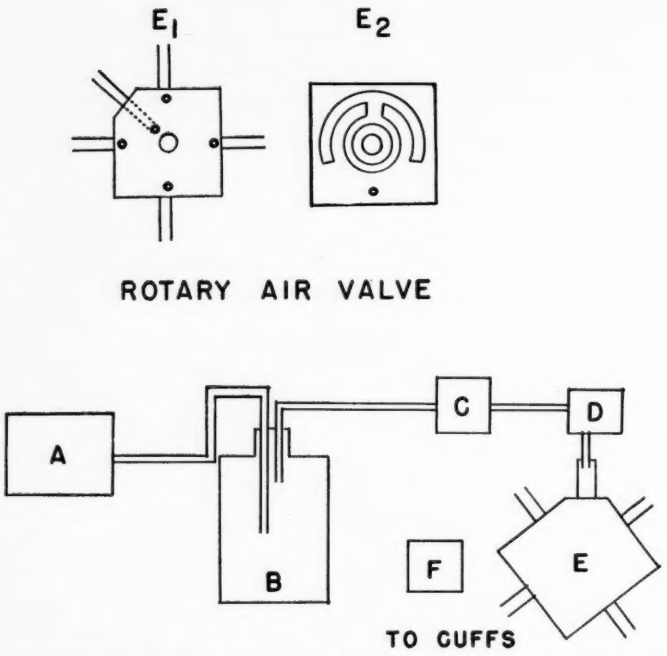

Fig. 1.—Diagram of automatic rotating cuff apparatus. *A*, Air compressor and 1/10 horsepower motor; *B*, air reservoir; *C*, pressure gauge (anaeroid); *D*, bleeder valve; *E*, rotary air valve; E_1 and E_2, details of rotary air valve; *F*, 45 minute clock motor which drives *E*.

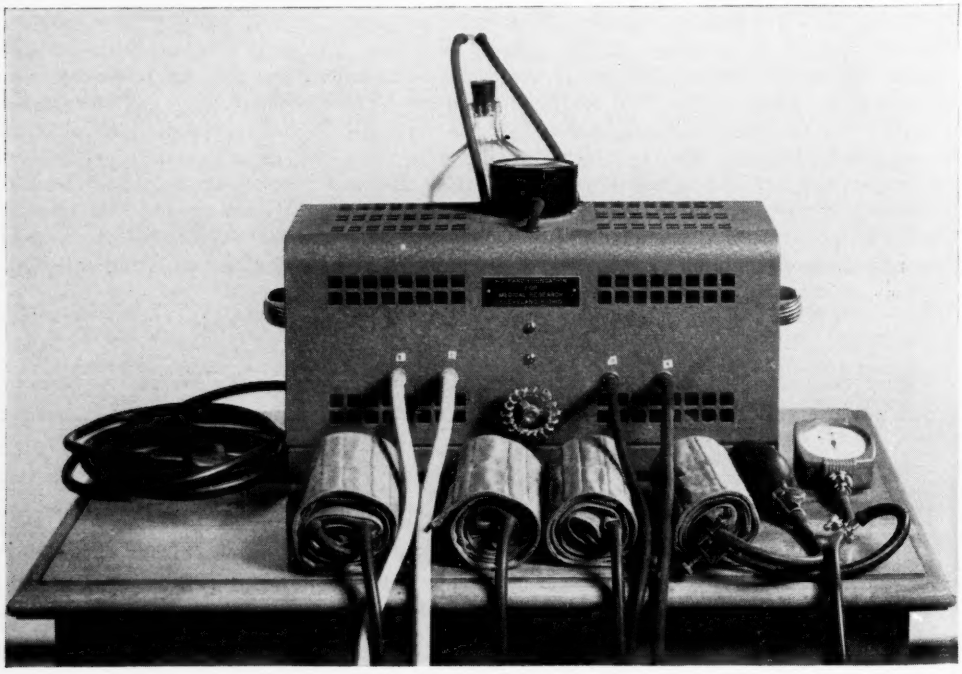

Fig. 2.—Photograph of automatic rotating cuff apparatus.

The choice of eleven-minute intervals for rotation instead of the customary fifteen minutes is arbitrary. The authors make no claim for the superiority of either interval.

A two-way stopcock is attached to one of the arm cuffs to permit determination of arterial blood pressure with a sphygmomanometer.

The air reservoir permits a more even pressure head to be applied to the cuff. During the inflated period, the pressure varies less than 2 mm. Hg even for periods of continuous operation lasting twenty-four hours.

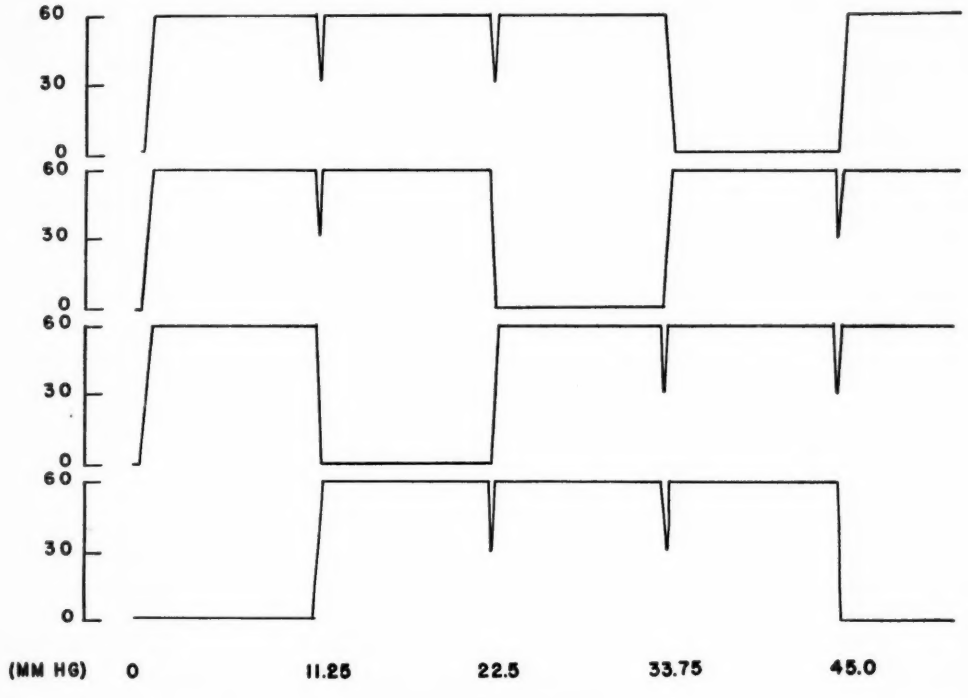

Fig. 3.—Pressure changes in four blood pressure cuffs during operation of automatic rotating cuff apparatus (discussed in text).

METHOD OF USE

The four cuffs are applied to the proximal parts of the extremities. (The rubber tubing from the cuffs to the apparatus is sufficiently long to allow the apparatus to be placed on a near-by table.) The pressure in the cuffs is regulated by the bleeder valve. A pressure of over 30 mm. Hg is built up in the system within twelve seconds of connecting the air compressor motor. The pressure should be above venous pressure and below systemic diastolic pressure. In our experience a pressure level of 35 to 50 mm. suffices.

The pressure gauge should be checked periodically, mainly to guard against leaks. In actual practice the air pressure has remained constant over long periods of operation. The use and control of the apparatus have been simple and fool-proof.

COMMENTS

This apparatus has been used on the private and staff services of the University Hospitals of Cleveland since 1951, as an adjunct in the treatment of more than one hundred patients with pulmonary edema and/or right heart failure. There have been no mechanical breakdowns. The length of time of

application of the apparatus has varied, from several hours to days. In a few unusual instances of recurrent pulmonary edema the uninflated cuffs remained applied for several days and the patient started the air compressor motor as indicated.

There has been no clinical evidence that venous thrombosis or arterial ischemia was produced in any instance.

The attending nurses and physicians have been particularly impressed by the ease of operation, safety, efficiency, and precise automaticity of the apparatus which permits the nurses to render other care to the patient during its use. On a busy service, especially at night, this economy of time is significant.

The apparatus is also particularly valuable in admitting and accident wards. It is portable and could be used in private home practice. Perhaps the present glass air reservoir should be replaced by a plastic or metal one for better portability.

SUMMARY

An automatic rotary cuff apparatus has been developed for use as an adjunct in the treatment of acute heart failure. The apparatus permits venous compression and minimizes the occurrence of venous thrombosis by partial deflation of all cuffs every eleven and one-quarter minutes.

A COMPARISON OF THE VOLUME OF DISTRIBUTION OF ANTIPYRINE, N-ACETYL-4-AMINO-ANTIPYRINE, AND I^{131}-LABELED 4-IODO-ANTIPYRINE IN HUMAN BEINGS

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THE measurement of total body water is of interest both to the clinician and to the clinical investigator. Although several methods have been devised for estimating the volume of body compartments, no simple and rapid technique for measuring body water has been generally available. From a theoretical standpoint, deuterium as D_2O or tritiated water have been regarded as the ideal tools for this purpose[1-4]; however, the measurement of deuterium and of tritium require instrumentation which is beyond the reach of many laboratories.

Despite certain theoretical objections, antiyprine has been widely used for the measurement of body water; however, its chemical determination requires the use of an ultraviolet spectrophotometer, and sample preparation, together with the measurement of optical density, becomes time-consuming. More recently, a derivative of antipyrine, N-acetyl-4-amino-antipyrine has been utilized for the estimation of body water. This method has an advantage over antipyrine in that the determination can be carried out colorimetrically in the visible range of the spectrum.[5]

Although these several methods have been applied to the study of body water under a variety of conditions, a simple and rapid method utilizing isotopic tracers seemed desirable. Preliminary studies in dogs demonstrated that 4-iodo-antipyrine distributed itself in a volume approximately the body water.[6] The current study was therefore undertaken to compare the volume of distribution of antipyrine, N-acetyl-4-amino-antipyrine, and I^{131}-labeled 4-iodo-antipyrine in human beings.

METHODS

The 4-iodo-antipyrine was prepared by the method of Emery and Palkin,[7] and was labeled with I^{131} by means of an exchange reaction with Na I^{131}. Excess I^{131} was removed by passing the compound through a column of anion exchange resin. The purity of the compound was checked by ascending strip-paper chromatography using 50 per cent ethanol-toluene as a solvent. Under these conditions the iodide remains at the point of origin and the 4-iodo-antipyrine moves to Rf = 0.95. Each batch of 4-iodo-antipyrine was checked in this fashion and it was found that 100 per cent of the I^{131} was chemically bound to the parent compound.

From the Department of Medicine of the Stritch School of Medicine of Loyola University; The Mercy Hospital, Chicago, Ill., and the Department of Radioactive Pharmaceuticals, Abbott Laboratories, North Chicago, Ill.

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The subjects for this study included 16 normal individuals who were free of edema. All studies were performed in the postabsorbtive state and only liquids were permitted by mouth until eight hours after the time of injection. After a control blood sample had been drawn, 50 μc of I^{131} as 4-iodo-antipyrine,* 750 mg. of N-acetyl-4-amino-antipyrine,† and 1,000 mg. of antipyrine,‡ were administered intravenously from an infusion flask. (The various substances were measured and delivered into the infusion flask with sterile, calibrated syringes.) The infusion flask was then rinsed five times with 5 per cent glucose solution (previous studies had demonstrated that this number of rinses was required to assure delivery of the total amount of material in the infusion flask). All subjects emptied their bladders prior to the time of injection and a 24-hour urine collection was obtained in order to determine the amount of 4-iodo-antipyrine excreted in 24 hours.

Samples of venous blood were withdrawn 3, 4½, and 6 hours after the time of injection. In two subjects, blood samples were also drawn 5, 10, 15, 30, 60, and 120 minutes after the injection. After clotting, the samples were centrifuged and the serum removed for analysis.

Assays for radioactivity were made on 1 ml. aliquots of serum using a well type scintillation counter and a single channel pulse-height analyzer. All counts were corrected for background and radioactive decay. Thyroid uptake after 24 hours was evaluated by external counting over the thyroid area with a probe type scintillation counter.

Analyses for antipyrine were performed on zinc filtrate of serum according to the method of Soberman.[8] A Coleman Universal spectrophotometer supplied with a specially selected photo cell and a PC3 filter was utilized for the measurement of optical density. Analyses for N-acetyl-4-amino-antipyrine were performed following the method of Brodie.[5]

The volume of distribution for each of the substances was calculated by plotting the concentration of the various compounds against time on semilogarithmic paper and then extrapolating back to determine the concentration at zero time. This extrapolated value was then corrected for serum water content by dividing by 0.93. The volume of distribution of each substance was then determined by dividing the amount injected by the corrected zero-time value.

In order to evaluate the importance of thyroid uptake on the volume of distribution of the 4-iodo-antipyrine, four subjects were pretreated with Lugol's solution, 10 drops four times daily for two days prior to the test.

Evidence has been presented suggesting that antipyrine is reversibly bound to plasma and tissue proteins.[8] In the present studies a large quantity of antipyrine (as compared with the amount of 4-iodo-antipyrine) was administered to each subject, and, as a result, the possibility of competitive protein binding needed consideration. Therefore, in four cases the 4-iodo-antipyrine was administered alone on one day and its volume of distribution was compared with the volume of distribution of antipyrine and N-acetyl-4-amino-antipyrine determined on the following day.

RESULTS

The results are shown in Table I and Fig. 1. The mean value for the volume of distribution of antipyrine was 31.6 kg. or 49.7 per cent of the body weight, for N-acetyl-4-amino-antipyrine was 31.9 kg. or 50.6 per cent of the body weight, and for 4-iodo-antipyrine 31.9 kg. or 50.6 per cent of the body weight. The differences between these values were not statistically significant.

The mean value for the 24-hour thyroid uptake was 7.9 per cent of the injected dose. In the four individuals pretreated with Lugol's solution the mean thyroid uptake was 2 per cent. The mean value for 24-hour urinary excretion of

*Supplied by Abbott Laboratories, North Chicago, Ill.
†Supplied by Irwin Neisler and Company, Decatur, Ill.
‡Supplied by Eli Lilly and Company, Indianapolis, Ind.

TABLE I. THE VOLUME OF DISTRIBUTION OF ANTIPYRINE, N-ACETYL-4-AMINO-ANTIPYRINE, AND 4-IODO-ANTIPYRINE

PATIENT	AGE (YR.)	WEIGHT (KG.)	VOLUME OF DISTRIBUTION AP 1 (KG.)	PER CENT 2	NAAP 3 (KG.)	PER CENT 2	4 I-AP 4 (KG.)	PER CENT 2
A.								
H. A.	65	88.2	42.1	47.7	44.5	50.4	44.2	50.1
C. D.	29	61.4			34.7	56.4	35.8	58.1
W. S.	27	62.7	31.2	49.8	34.2	54.6	31.3	49.9
J. S.	26	64.5	34.1	52.8	37.3	57.8	34.2	53.0
M. J.	36	67.3	37.2	55.3	37.0	54.9	37.4	55.5
J. M.	27	80.9	34.1	42.2	36.3	44.9	34.8	43.0
J. W.	27	76.3	37.8	49.5	37.3	48.9	37.1	48.6
J. B.	41	64.1	31.4	48.9	31.4	48.9	31.9	49.8
H. W.	44	81.3	36.5	44.5	37.0	45.5	39.0	48.0
A. Y.	28	70.5	38.7	54.9	36.7	52.1	37.2	52.8
I. H.	56	66.7	25.2	37.8	26.1	39.1	27.9	41.8
L. McK.*	39	42.3	21.4	50.6	17.5	41.4	17.1	40.4
B.								
B. P.*†	52	62.3	31.8	51.0	30.9	49.6	30.5	49.0
G. W.*†	30	45.5	24.8	54.5	26.6	58.4	26.5	58.2
A. P.*†	26	66.4	32.6	49.1	32.9	49.5	32.9	49.5
W. K.†	53	58.6	28.7	49.0	28.5	48.6	29.9	51.0
Mean A		=	33.6	48.5	34.2	49.6	34.0	49.3
S.D.		=	±6.1	±5.3	±6.8	±6.0	±6.7	±5.4
Mean B		=	29.5	50.9	29.7	51.5	29.9	51.9
S.D.		=	±3.5	±2.6	±2.8	±4.6	±2.6	±4.3
Mean A and B		=	31.6	49.7	31.9	50.6	31.9	50.6
S.D.		=	±3.6	±1.7	±3.2	±1.3	±2.9	±1.8

*Pretreated with Lugol's solution.
†4 I-AP and AP and NAAP determined on different days.
1 = Antipyrine space in kilograms.
2 = Per cent of body weight.
3 = N-acetyl-4-amino-antipyrine space in kilograms.
4 = 4-iodo-antipyrine space in kilograms.

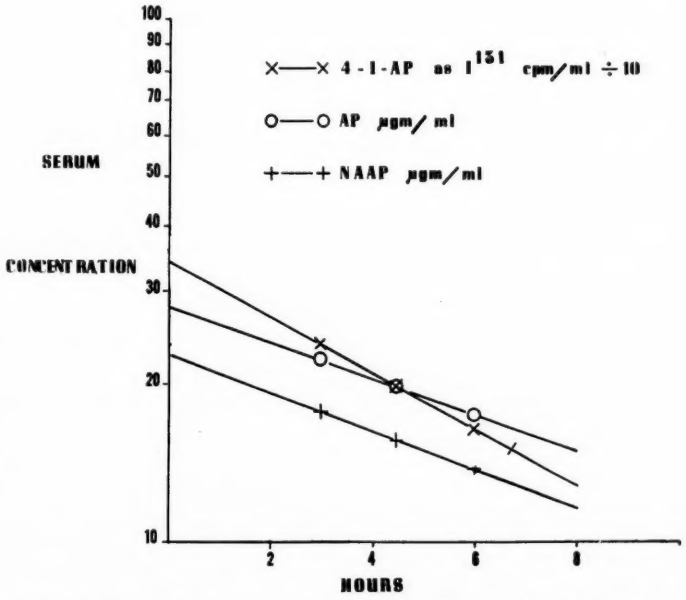

Fig. 1.—The serum disappearance curves for 4-iodo-antipyrine (4IAP), antipyrine (AP), and N-acetyl-4-animo-antipyrine (NAAP) in Subject W. S.

I^{131} was 71 per cent of the injected dose. The $C_{½}$ time for serum or the time required for the serum concentration to decrease by one-half after equilibrium had been reached had a mean value of 5.7 hours.

Fig. 1 illustrates the serum disappearance curves for antipyrine, N-acetyl-4-amino-antipyrine, and 4-iodo-antipyrine in a typical case.

DISCUSSION

Isotopic labeling of test compounds has been utilized in the past to facilitate the measurement of certain bodily functions or compartments. I^{131}-labeled human serum albumin[9] and thyroxine,[10] as well as S^{35}-labeled methionine,[11] are but a few examples. Although both the stable and radioactive isotopes of hydrogen have been available as tracers for body water for some time, their application has been limited because of the technical problems relating to their determination. By tagging a test substance with a gamma-emitting radioisotope, a rapid means for measuring this substance with a scintillation type counter becomes available.

In the present study, a derivative of antipyrine, 4-iodo-antipyrine has been tagged with I^{131} and its measurement thereby greatly simplified. Having produced a labeled substance it is then necessary to demonstrate that the functional characteristics of the parent compound have not been altered by the introduction of the isotopic label and that the label itself does not become detached from the compound. In the studies reported here the volume of distribution of I^{131}-labeled 4-iodo-antipyrine has been compared with closely related substances whose behavior and volume of distribution are known. Since the volume of distribution of 4-iodo-antipyrine is identical with that of antipyrine and N-acetyl-4-amino-antipyrine, it can be concluded that the isotope-labeled compound may be utilized to measure the antipyrine space and with the reservations which apply to the antipyrine space, the 4-iodo-antipyrine space may be interpreted as representing the total body water. The values for body water obtained in the current study correspond closely with those of other workers.[2-4]

The 24-hour uptake of I^{131} from 4-iodo-antipyrine was 8 per cent in subjects who had received no pretreatment with Lugol's solution. This amount of uptake apparently did not interfere significantly with the volume of distribution of the substance during the first 8 hours after injection; however, this can be reduced even further as illustrated by the fact that pretreatment with Lugol's solution reduced the thyroid uptake to only 2 per cent.

The very rapid disappearance of the compound from the serum ($C_{½}$ = 5.7 hours) and the rapid urinary excretion of the substance (71 per cent in 24 hours) reduces radiation hazard and makes feasible the serial determination of the 4-iodo-antipyrine space.

SUMMARY AND CONCLUSIONS

The volume of distribution of antipyrine, N-acetyl-4-amino-antipyrine and 4-iodo-antipyrine has been compared in 16 normal individuals. The values obtained were 31.6 kg. or 49.7 per cent of the body weight, 31.9 kg. or 50.6 per cent of the body weight, and 31.9 kg. or 50.6 per cent of the body weight, re-

spectively. The 24-hour thyroid uptake was measured and found to be 8 per cent of the injected dose. This did not appear to interfere with the volume of distribution of 4-iodo-antipyrine during the first 8 hours after injection, but could be further reduced by pretreatment with Lugol's solution. Under these circumstances urinary excretion of I^{131} is extremely rapid, 71 per cent of the injected dose being excreted in the first 24 hours.

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IN VIVO DEMONSTRATION OF THE LUPUS ERYTHEMATOSUS PHENOMENON

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THE demonstration of the lupus erythematosus phenomenon has heretofore been considered to be an in vitro finding. Eppes and Ludovic[1] studied the blood films made directly from finger puncture of numerous patients but found only one "neutrophilic leukocyte which appeared to be engulfing amorphous pink material." Hargraves[2] and Harvey and associates[3] have stressed the inability to demonstrate the lupus phenomenon in vivo.

The unique report of Chomet and his co-workers[4] described L. E. cells in blood films obtained from a terminally ill patient with lupus erythematosus several days before death. Our technique of demonstrating the L. E. phenomenon in vivo was an outgrowth of their report as well as of the observation by Haserick[5] that leukocyte clumping appeared in five or six minutes and that the L. E. phenomenon was evident after 12 or 13 minutes of incubation in vitro. Inasmuch as anoxia and stasis were assumed to be present in the terminally ill patient, these conditions were reproduced by constriction in a finger.

MATERIAL

The technique to be described was applied in the study of 23 patients with known disseminated lupus erythematosus and 50 controls. None of the patients was severely ill; 21 were ambulatory.

METHOD

A rubber band placed around a digit with sufficient tightness to produce cyanosis is left on for 20 minutes. At the end of this time, blood films are made from simple finger puncture before the constriction is released. The film is made somewhat thicker than is customary in order to facilitate the search for both rosettes and L. E. cells. The slides are stained in the usual manner; however, we have found May-Greenwald and Giemsa stain to be superior to Wright's stain, because a brighter color is produced by the amorphous mass of the L. E. cell. Two or three slides should be prepared and studied carefully for fully ten minutes with the low-power objective, before the slides are pronounced negative.

RESULTS

The preparations of both the 23 patients with known lupus erythematosus and the 50 controls were studied for ten-minute periods. The examinations were considered positive when two or more typical L. E. cells could be found. L. E.

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cells were readily observed in 18 of the 23 patients; in 16 of these, rosettes were also seen. Rosettes were also identified in two of the five patients who failed to show the in vivo L. E. phenomenon. It is of interest that although all five of these patients previously had been found to have L. E. cells by the clot technique, when the negative in vivo tests were obtained, two patients also failed to show the phenomenon in vitro.

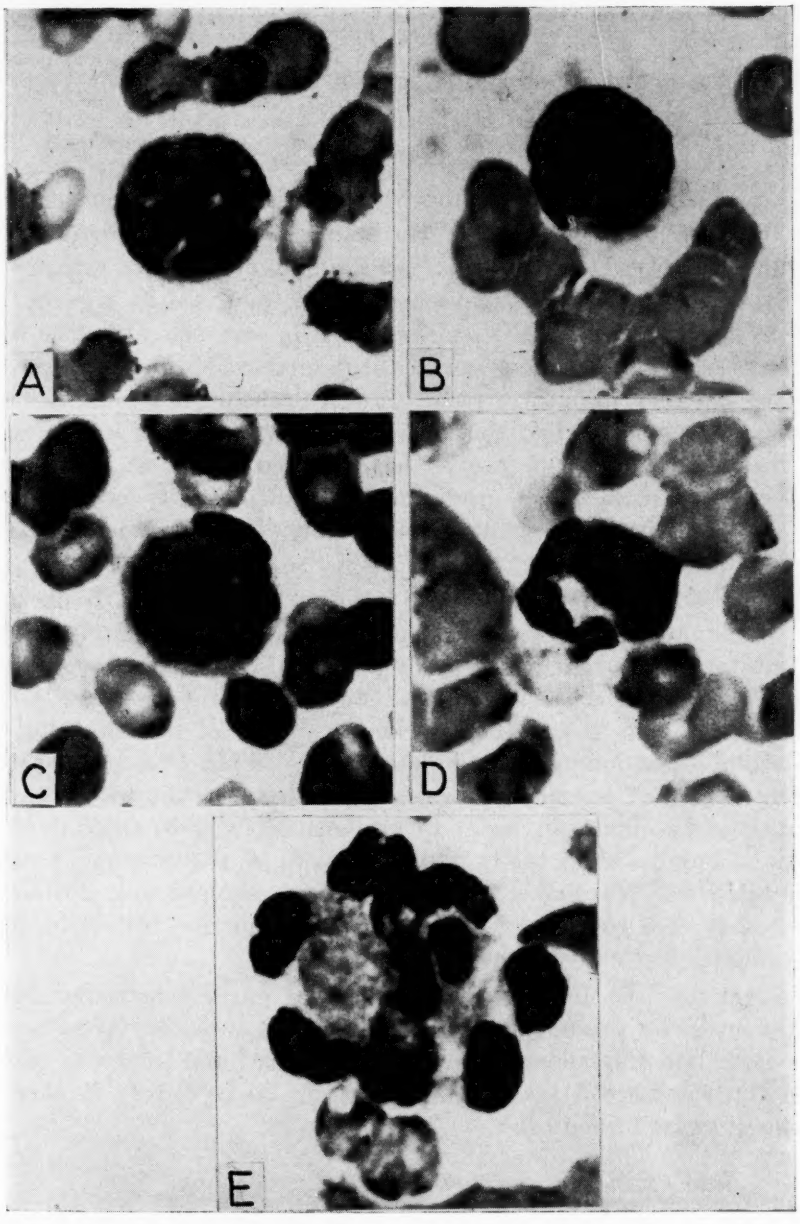

Fig. 1.—*A*, *B*, *C*, and *D*, Representative L. E. cells; *E*, rosettes as seen in patients with lupus erythematosus tested by technique described.

Notwithstanding the occasional severe leukopenia, the L. E. cells were found without too much difficulty when the preparations were positive. Blood films obtained without constriction, simultaneously with the blood films from the constricted fingers, did not reveal L. E. cells.

The white blood cell count and hemoglobin values in the constricted and unconstricted digits were compared in nine of the patients. The only essential difference found was in one case in which the white cell count was higher with constriction.

In the control group, occasional tart cells, but no L. E. cells or rosettes, were seen.

DISCUSSION

The method presented is the first to elicit the L. E. phenomenon in vivo with a fair degree of consistency. The method has the advantages of directness and simplicity. On the other hand, apparently the in vivo method is somewhat less sensitive, as might be expected, than the in vitro test. The failure to demonstrate the L. E. phenomenon in vivo should not be taken as proof that the L. E. factor is absent without further attempts at elicitation by the use of the various in vitro tests. It will probably be found, as more experience is accumulated, that considerable fluctuation occurs in the ease with which the L. E. phenomenon can be demonstrated in vivo as well as in vitro. There seemed to be no way to grade the degree of positivity or to compare the in vivo or the in vitro tests quantitatively. This was not unexpected in view of the uncertainties of the test.

The mechanism responsible for the demonstration of the L. E. phenomenon in vivo is unknown. Several obvious possibilities suggest themselves: anoxia, stasis, substances associated with the liberation of factors due to vascular constriction, exposure of cells to prolonged contact with the vessel walls, changes of pH, or the death of cells due to one or several of the foregoing causes. Fundamental causation remains undetermined. What effects time, temperature, and degree of vascular obstruction will have on the test, both from a qualitative and a quantitative standpoint, must likewise be ascertained before the optimal conditions for the test will be known, so that maximum sensitivity may be achieved. No further evidence has been adduced to indicate that the in vivo test is more specific or less specific than the in vitro test in the diagnosis of disseminated lupus erythematosus.

We feel that the finding of rosettes by the finger constriction technique probably justifies a presumptive diagnosis of acute lupus erythematosus, since rosette formation was absent in every control case, and since two of the five lupus erythematosus patients who failed to show the in vivo L. E. phenomenon did display rosette formation.

SUMMARY

1. A method for the demonstration of the lupus erythematosus phenomenon in vivo is described.

2. This method was applied to 23 cases of known lupus erythematosus and the results were found to be positive in 18.

3. Fifty control patients gave negative results.

4. The test is suggested as a simple screening technique for the detection of the lupus erythematosus phenomenon.

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LABORATORY METHODS

DESCENDING CHROMATOGRAPHIC BEHAVIOR AND DIFFERENTIATION OF SOME ANTIHISTAMINICS AND ALKALOIDS

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INTRODUCTION

DURING the course of many criminal investigations at this laboratory we have had occasion to examine materials in the form of capsules, tablets, and body fluids for narcotics. A surprising number of these substances have been shown to contain antihistaminics. In five of these cases investigated, the addicts were using the antihistaminics for direct intravenous injection.

Thus it became necessary to devise some method for the rapid yet simple determination of the antihistaminics as well as their differentiation from the alkaloidal narcotics and poisons. The problem of differentiation is particularly difficult due to the identical reaction given by many of the antihistaminics and alkaloids with the common alkaloidal spot reagents, e.g., Marquis', Frohde's, and Buckingham's. It was felt that many small laboratories dealing with medicolegal materials might be badly led astray or confused by these conflicting reactions.

Descending chromatography was chosen as the tool for analysis because of the small sample required and the number of tests possible after chromatographic analysis is complete. The general developing spray chosen was the potassium iodoplatinate reagent, [1-3] because of its characteristic reaction with all of this group of tertiary amines. Supplementary information was taken from the ultraviolet absorption characteristics of these compounds.

MATERIALS AND METHODS

Developing Solvent Pairs.—The n-butyl alcohol used in these pairs was Mallinckrodt Analytical Reagent; 1.00 normal hydrochloric acid was prepared volumetrically from constant boiling acid; pH 6.5 buffer was prepared according to Sorensen and was adjusted to exactly pH 6.5 using a Beckman pH meter. All developing solvents should be allowed to equilibrate, in the room used for analysis, for three days prior to use.

n-Butyl Alcohol—1.00 N HCl solution (Developing solvent No. 1).—Saturate n-butyl with 1.00 normal HCl.

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The statements herein expressed do not necessarily represent the opinions of the Office of the Surgeon General or the Department of the Army.
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n-Butyl Alcohol—1.00 N HCl solution (Developing solvent No. 2).—Ten milliliters of 1.00 normal HCl are added with an accurately calibrated volumetric pipette to a 100 ml. volumetric flask; dilute to volume with n-butyl alcohol and shake until dissolved. There should be no trace of clinging droplets of water on the sides or bottom of the flask.

n-Butyl Alcohol—pH 6.5 Phosphate Buffer (Developing solvent No. 3).—Shake 400 ml. n-butyl alcohol with pH 6.5 phosphate buffer in a 500 ml. separatory funnel until completely saturated.

Butyl Alcohol-Water Mixture (Atmosphere solvent pair).—Saturate 800 ml. of n-butyl alcohol with distilled water, for each developing jar used. Allow to equilibrate three days before use. New atmosphere solvent should be used for each run.

Cyanogen Bromide Crystals.—Eastman Kodak #919 (reagent of Huebner[4]).

Benzidine Alcohol Mixture.—Add 0.250 Gm. benzidine to 500 ml. of 50 per cent alcohol in a 100 ml. volumetric flask. Shake until dissolved and dilute to volume (reagent of Huebner[4]).

Iodoplatinate Reagent.—Five milliliters of 5 per cent platinic chloride in 1 N hydrochloric acid, 45 ml. of 10 per cent potassium iodide and 100 ml. of distilled water (stored in a brown glass bottle).

Kieffers' Reagent ($K_3Fe[CN]_6 + FeCl_3 \cdot 6H_2O$).—Mix 50 ml. of a 1:100 fresh solution of potassium ferricyanide with 50 ml. of 10 per cent ferric chloride. Use the same day as prepared.

Sorensen's Phosphate Buffer pH 6.5.—Adjust to exactly pH 6.5 using a pH meter.

1.00 N Hydrochloric Acid.—Prepared volumetrically from constant boiling acid.

0.5 N Hydrochloric Acid.—Prepared volumetrically from constant boiling acid.

Saturated Sodium Carbonate.

Saturated Potassium Dihydrogen Phosphate.

Saturated Sodium Borate pH 9.0.

2 per cent Sodium Sulfite in Saturated Sodium Borate.

Chloroform U.S.P. washed with 0.15 N HCl.

0.5 N NaOH (Volumetric).

Chloroform Isobutyl Alcohol (General extracting solvent).—Eighty milliliters of chloroform to 20 ml. of isobutyl alcohol.

Chromatography Paper.—Whatman No. 4 special for chromatography (W & R Balston Limited, London, England) 57 × 47 cm.*

Chromatography Jars.—Each setup consists of 2 large museum jars (45 cm. length by 47 cm. diameter) placed mouth to mouth. The upper jar has a small hole 2 cm. in diameter drilled in its base, with a rubber stopper and glass tube for the addition of the solvent pair (Fig. 1).

Butter Dish Developing Boats.—Each setup has as a developing boat, a small plastic butter dish measuring 14.8 cm. by 5.7 cm. and 3.5 cm. deep. Glass rods, measuring 14 cm. in length and 1 cm. in diameter, are attached to the long sides of the dish in the manner shown in Fig. 2. The developing boat is placed on the glass support in the manner shown in Figs. 1 and 2.

Unknown Tablets, Capsules and Powders.—

Ten to fifty milligrams of the substance (all of the weighed substance if less than 10 mg.) (note weight), dissolved in a small amount of 0.5 N HCl (approximately 2 to 5 ml).

Adjust to pH 9.0 (with pH Hydrion paper) by the addition of saturated sodium carbonate.

Extract with 2 volumes of the general solvent.

Filter through coarse Whatman No. 40 filter paper and evaporate to dryness in a beaker containing a small amount of 1.0 N HCl (4 to 5 drops).

The residue in the beaker is taken up in a measured volume of absolute alcohol. Note volume.

*Obtained from Scientific Supplies Company, Seattle, Wash.

Fig. 1.—Manner in which the chromatogram jars are placed in conjunction with each other and placement of chromatograms in the butter dish boats.

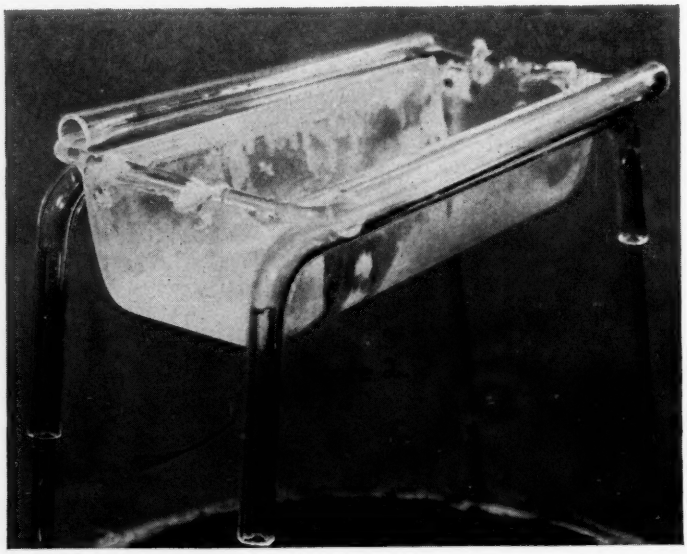

Fig. 2.—Plastic butter dish and proper placement of glass rods in relationship to boat.

The residue is now ready for chromatographic analysis.

Urine and Blood Extraction (modification of Goldbaum and Kazyak[2]).—

One-half the volume of a 100 to 200 ml. submitted urine sample is placed in each of two 250 ml. Erlenmeyer flasks labeled "A" and "B".

Flask A is made acid with a 1:10 volume of concentrated hydrochloric acid and autoclaved for 30 minutes at 15 pounds steam pressure.

Flask B is adjusted to pH 4 with 1.0 N hydrochloric acid (this flask is not autoclaved).

After 30 minutes Flask A is removed from the autoclave and both Flasks A and B are adjusted to pH 9.0 by the addition of saturated sodium carbonate.

Contents of Flasks A and B are placed in Squibb separatory funnels and extracted with 3 volumes of chloroform-isobutyl alcohol solvent, general solvent mixture.

The general solvent mixture layer is extracted with 1 volume of 0.5 N hydrochloric acid.

The hydrochloric acid phases are placed in Erlenmeyer flasks labeled "A" and "B," respectively. The acid phases are again adjusted to pH 9.0 with saturated sodium carbonate and re-extracted with 3 volumes of the general extracting solvent.

The organic solvent phases are filtered through Whatman No. 40 filter paper into beakers labeled "A" and "B" containing 4 to 5 drops of 1.0 N HCl.

The filtrates are evaporated to dryness on a steam bath and taken up in a measured volume of absolute alcohol. The substance is now ready for chromatographic analysis.

The *method for blood* is essentially that for urine. Twenty to thirty milliliters of whole blood are diluted with an equal volume of distilled water, one-half is placed in a flask labeled "A" and one-half in a flask labeled "B".

Proceed as for urine, paragraph 2, et seq.

Application to Tissue Homogenates (modification of Mannering, Dixon, Carroll and Cope[1]).

One hundred grams of tissue, homogenized in a Waring blendor, is adjusted to pH 4.0 with saturated potassium dihydrogen phosphate.

Autoclave for 30 minutes at 15 pounds pressure, squeeze while hot and save aqueous extract.

Add 2 ml. of phosphoric acid (concentrated) to acid aqueous extract.

Extract with 3 volumes of chloroform (if emulsion forms break by centrifuging).

Adjust aqueous phase to pH 9.0 with saturated sodium carbonate.

Transfer to Squibb separatory funnel and extract with 3 volumes of isobutyl alcohol-chloroform mixture.

Filter and proceed as for urine, paragraph 6, et seq.

Chromatographic Analysis.—

A small measured volume (0.01 ml.) of the alcoholic extracts from above is taken up in graduated capillary pipettes and placed on filter paper and sprayed lightly with the iodoplatinate reagent, employing an ordinary atomizer. If the typical blue-black iodoplatinate reaction is not produced, the test is considered negative (alkaloids and/or antihistaminic compounds are not present).[3] If positive, proceed with the chromatographic analysis, as follows:

Six paper strips are cut from the Whatman No. 4 special chromatographic paper; each strip is 57 cm. in length and 12.5 cm. in width. The long sides of the paper strips run in the indicated machine direction of the paper.

Four of the papers are washed thoroughly with 1.0 N HCl[3, 5] by running four times through a trough containing the acid and then hung up to dry. The papers are allowed to dry until the last trace of HCl has disappeared.

Two of the papers are equilibrated with pH 6.5 buffer solution by running through a chemically clean trough containing the buffer[3, 5] four times and hung up to dry. Drying of the pH 6.5 papers should take place entirely separate from the drying HCl papers to prevent reaction of the volatile HCl on the pH 6.5 buffered strips.

CAUTION: Papers used in this analysis should be handled as little as possible, preferably with clean rubber gloves. All papers should be run smoothly through the wash solution to avoid salt concentration or unequal washing of the paper.

TABLE I. RF VALUES, SPOT TESTS AND ULTRAVIOLET DATA FOR SOME TERTIARY AMINES. $Rf = \frac{\text{COMPOUND FRONT}}{\text{SOLVENT FRONT}}$

COMPOUND	DEVELOPING SOLVENT 1	DEVELOPING SOLVENT 2	DEVELOPING SOLVENT 3	FLUORESCENCE BEFORE CNBr	FLUORESCENCE AFTER CNBr	CNBr + BENZIDINE	$K_3Fe(CN)_6 + FeCl_3 \cdot 6H_2O$	U.V. SPECTRA IN 0.5 N HCl	
								MAXIMA	MINIMA
Benadryl	Rf .964	Rf .952	Rf .926					260	245
Chlor-Trimeton	.65	.54	.94			P 50 (S)		265	218
Decapryn	.35	.22	.89			P 50 (S)		260	235
Dramamine	.96	.94	.96		SF 50 (S)	P 50 (S)		260	245
Neo-Antergan	.47	.326	.93	F 10 (S)	F 10 (S)	Y 50 (S)		240	260
Neohetramine	.47	.35	.89	F 15 (S)	F 5 (S)	Y 50 (S)		235(275)	260
Perazil	.956	.90	.988					230	218
Pyribenzamine	.417	.328	.948	F 5 (S)	F 5 (S)	Y 50 (S)		240(315)	260
Thenylene	.36	.249	.924	F 20 (S)	F 10 (S)	Y 50 (S)		236(315)	260
Thephorin	.96	.952	.968			Y 50 (S)		264	240
Trimeton	.387	.22	.864			P 40 (S)		265	235
Atropine	.828	.782	.624					257	244
Codeine	.582	.57	.479					285	260
Cocaine	.862	.842	.844					235	None
Heroin	.484(.71).79	.40(.68).75	.61(.79)				B 10 (S)	280	255
Demerol	.936	.907	.908					257	248
Dilaudid	.544	.459	.307				B 10 (S)	280	261
Dionin	.782	.76	.714				B 10 (S)	285	260
Homatropine	.732	.67	.466					257	247
Morphine	.50	.44	.32				B 5 (S)	285	260
Methadone	.954	.95	.95					260(293)	250(275)
Papavarine	.874	.85	.984	F 20 (S)	SF 10 (S)	O 20 (S)		251(285)308	269(290)
Pilocarpine	.389	.286	.66					None apparent	
Procaine	.17	.11	.63				B 5 (S)	228(279)	255
Quinacrine	.894	.872	.82	F 15 (S)	F 20 (S)			280	242
Scopolamine	.60	.548	.65					257	244
Strychnine	.80	.76	.618					254	228

RF values obtained using the method outlined and sensitivities of tests used for identification of the compounds, with expected values for the individual test or groups of tests. Ultraviolet maxima and minima are given for each compound chromatographed.

(S)=Sensitivity in μg per 4 mm. spot; P=Pink; Y=Yellow; O=Orange; B=Blue; F=Fluorescence; SF=Slight Fluorescene.

After the papers are dry, lines are drawn 13 cm. from one end of the strips and 5 cm. from the opposite ends. Strips are labeled "6.5 saturated," "HCl saturated," and "10 ml. 1.00 N HCl per 100 n-butyl alcohol."

Unknowns and standards, if desired, are placed on the 13 cm. line, 2.5 cm. from the edge and 2.5 cm. from each other. The amount should be recorded for quantitative analysis. The amount will vary with the apparent concentration of the initial iodoplatinate spot. Spots should be as close to 4 mm. in diameter as possible. If the area of application is not uniform for each spot, marking of the true head of the spot will be difficult and spot area quantitation will be impossible.

Each set of two papers is punched with three equidistant holes at the extreme 13 cm. end of the strips with a paper punch and are clamped together by threading a small glass rod through the punched holes.

The clamped ends of the papers are placed in the small butter dishes (boats) and weighted down with heavy glass rods.

The boats and papers are placed on the glass supports in the lower chromatogram jars, the upper jar is put in place and the juncture of the two is sealed with modeling clay (Fig. 1).

Glass tubes are run down into the chromatogram jars through rubber stoppers. The ends are approximately 2 cm. from the papers in the boats. The bottles are placed over the upper opening of the glass tubes and are sealed with clay.

The lower jar of each set-up contains 800 ml. of the n-butyl alcohol saturated with water (atmosphere solvent).

The papers are allowed to equilibrate with the atmosphere for exactly fourteen hours (according to Schute[5]). Proper timing of equilibration period and developing time is essential for reproducibility of results.

At the end of fourteen hours the bottles are removed from the upper jars and 100 ml. of the appropriate developing solvent pair is allowed to flow evenly down the glass tube.

The bottles are replaced and sealed with clay.

The solvents are allowed to advance until their fronts are at the 4 cm. marks at the lower end of the papers (approximately 6 hours). This sequence is used so that the papers can be set in the jar for equilibration at the end of the working day and the developing solvent pairs added the morning of the next day.

The papers are removed from the jars and allowed to dry.

Papers are examined for flourescent spots using a Woods light in a dark room. The heads of these spots, if any, are marked, and the Rf's are calculated (Table I).

One of each pair of strips is placed in a tightly sealed museum jar containing crystals of CNBr (method of Huebner[4]). After 15 minutes the paper strips are removed from the jars and again observed for fluorescence. Mark the heads of any spots and calculate Rf's (Table I).

The papers are sprayed lightly with benzidine alcohol (reagent of Huebner[4]) (if sprayed heavily, displacement of the spots may occur and erroneous Rf's will result) and replaced in the CNBr jars for twenty minutes and are observed for characteristic color reaction (Table I).

The papers are now removed from the CNBr jar and allowed to dry.

The dried papers are sprayed with the iodoplatinate reagent and the spot fronts are marked and Rf's calculated (Table I).

$$\text{Rf} = \frac{\text{Measured compound front (head)}}{\text{Measured solvent front}}$$

One of the next set of papers is used for spot testing in the area of the spot found with its corresponding paper.

This is accomplished in the following manner: a cardboard is placed at the 4 cm. mark, and fine spraying with iodoplatinate is commenced, as the cardboard is moved down the paper strip, until a spot front is noted.

The rest of the spot is used for testing with the ferricyanide-ferric chloride reagent (Table I); this reagent is added as a light spray from an atomizer. The blue color should develop instantaneously. Any color developing after standing is due to oxidation of the reagents and should be disregarded.

This series of conditions should be adequate to identify a given compound. If, however, further identification and quantitation are desired, the following procedure is used.

The remaining two papers are marked with the exact position of the compound by use of the Rf values calculated from the previous two counterpart papers.

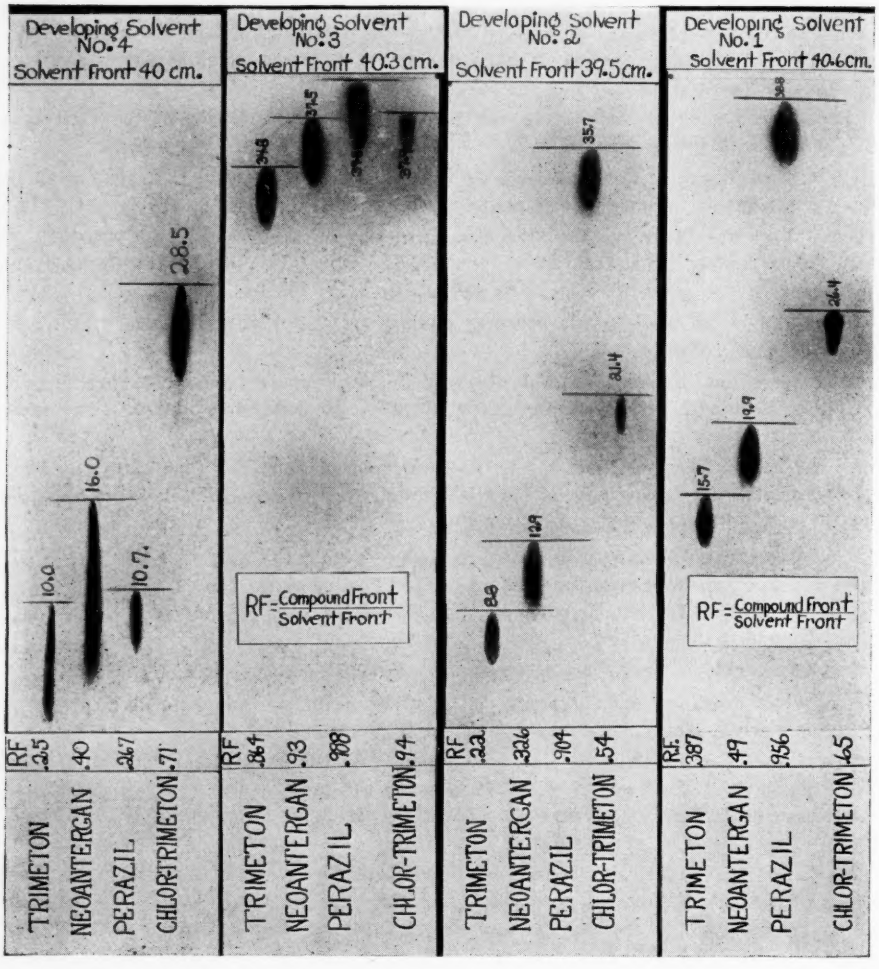

Fig. 3.—Four typical chromatograms obtained using the method outlined and with solvent pairs used. Developing Solvent 4 was prepared by saturating n-butyl alcohol with 50 per cent glacial acetic acid. This pair is used for comparison purposes and illustrates the innumerable solvent pairs possible. Note the very rapid movement obtained with the antihistaminics using developing Solvent 3.

This exact area is cut out, rolled and placed in a 50 ml. graduated cylinder containing saturated sodium borate pH 9.0 and then extracted with 25 ml. isobutyl alcohol-chloroform (if the compound is suspected from the chromatographic data to be morphine, the method of Goldbaum and Kazyak[2] may be used. The separated organic solvent is extracted with 4 ml. of 0.5 N HCl (a blank should be used with the same chloroform isobutyl volume as the sample and extracted with 4 ml. of 0.5 N HCl). The acid aqueous test layer is transferred

to a silica cell and read against the blank in the Beckman DU spectrophotometer in the range of 220 mμ to 320 mμ, advancing each reading 5 mμ. If an emulsion forms in the final extraction, it can be separated easily by centrifuging.

It is our practice in this laboratory to identify the compound by its very characteristic curve as well as its maxima and minima. This is done by plotting ultraviolet readings of the compound on thin tissue paper laid over blank graph paper. This tissue paper curve is then placed over all curves of the knowns with the same maxima and minima. (Table I.) *NOTE:* If desired, the paper in paragraph 24 can be complexed with iodoplatinate and extracted with

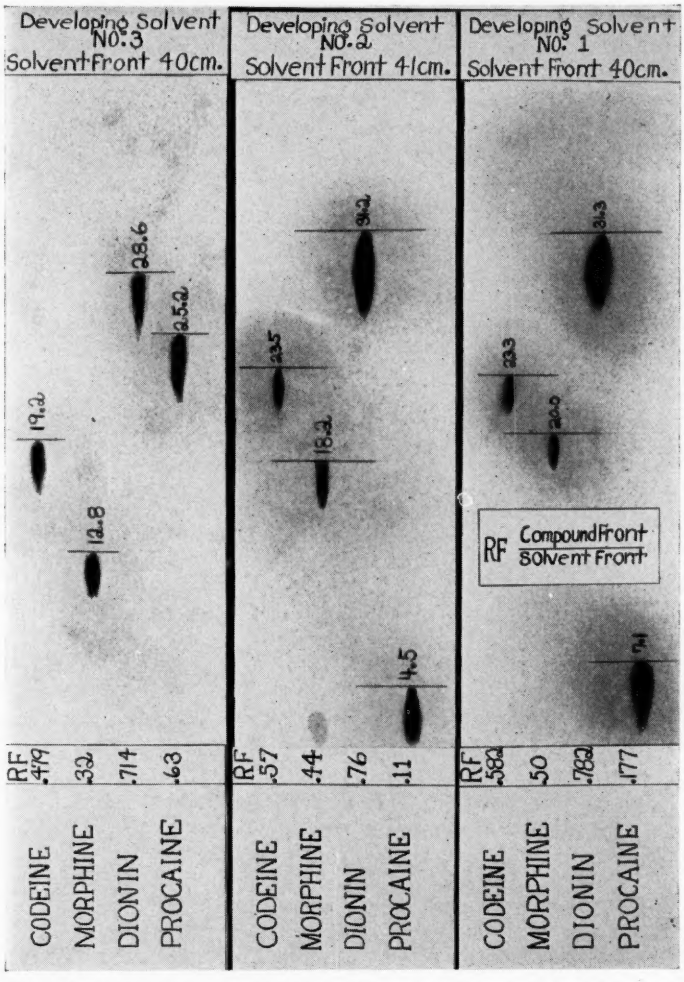

Fig. 4.—Typical chromatograms obtained with four other tertiary amines using the three solvent pairs shown. Note the comparatively slow movement obtained with developing Solvent 3 for these compounds as compared with the antihistaminics in Fig. 3.

2 per cent sodium sulfite in saturated sodium borate and treated exactly as outlined. This, in some cases, will lower the recovery and will increase the ultraviolet absorption background. It does, however, increase somewhat the confidence that one has all of the spot.

Quantitation.—

Semiquantitation can be accomplished rather easily by running known concentrations of the compound over the range desired to measure on chromatographs and comparing the area of the resultant spot. This method has proved quite effective in this laboratory.

If, however, exact quantitation is desired, the absorbance of the compounds at their maxima is taken and calculated from the following formula using the absorptivity of a standard.

$$\text{Micrograms of substance in cell} = \frac{\text{UA-BA} \times 4}{\text{AB}}$$

where the amount of 0.5 normal HCl used to extract the final solvent mixture is 4 ml.

UA—Absorbance at peak found in Table I.

BA—Background absorbance

AB—Absorptivity of 1 μg per 1 cm. light path of known pure substance at the peak wavelength.

NOTE: Standard curves can be run with known amounts of the compounds added to chromatogram strips, chromatographed, extracted, read in the spectrophometer and compared with the unknown.

RESULTS

The effect of decreasing the water phase in the HCl solvent pair is to markedly reduce the Rf's of the compounds. By controlling the aqueous component one can effectively reduce or increase the separation of the compounds, as can be seen by the graphs in Fig. 5.

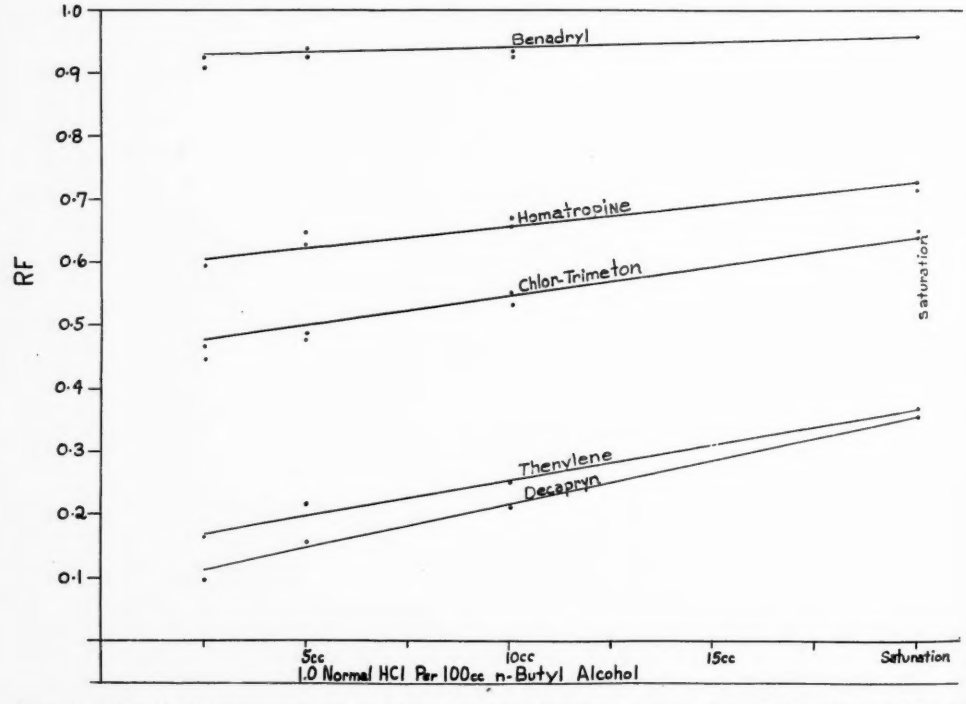

Fig. 5.—Results of increasing water phases on five teritary amines chromatographed. Each of the pairs used in this study was equilibrated for three days prior to its use.

Recoveries of most of the antihistaminics from urine were quite good, ranging from 46 to 86 per cent of ingested antihistaminics in human subjects over a 24-hour period (Table II).

TABLE II. QUANTITY OF ANTIHISTAMINICS RECOVERED IN MILLIGRAMS PER 24 HOURS AFTER INITIAL INGESTION, USING PAPER CHROMATOGRAPHY

COMPOUND	QUANTITY INGESTED (MG.)	QUANTITY RECOVERED USING U.V. DATA (MG.)	RECOVERY USING PAPER SPOTS (MG.)	U.V. RECOVERY (PER CENT)	SPOT RECOVERY (PER CENT)
Benadryl	50	38	40	75	80
Chlor-Trimeton	50	28.5	35	57	70
Decapryn	25	15.2	19.6	60.8	78.4
Dramamine	50	40.2	31.5	80.2	63
Neo-Antergan	50	37.6	31.2	75.2	62.4
Neohetramine	50	35.4	32.4	70.8	64.8
Perazil	50	38.6	30	77.2	60
Pyribenzamine	50	40.6	42.3	81.2	84.6
Thenylene	50	39.4	32	78.8	64
Thephorin	25	18.8	16.8	75.2	67.2
Trimeton	25	20.1	18.6	80.2	74.4

Results of recovery experiments after ingestion of varying amounts of the antihistaminics included in this study. All of the urine for a 24-hour period after initial ingestion was collected, extracted, and analyzed by the method outlined for urine.

Variations in recovery on identical samples were thought to be due to difficulty in reading spots as standards.

One case in which Benadryl had been the injected substance yielded 450 mg. per 500 ml. of urine sample. Benadryl was subsequently identified from the spoon and syringe used for injection of the substance.

Five cases were studied in which the patients were stockade prisoners. The subjects had been given Pyribenzamine "because they could not sleep." They had accumulated a large quantity of the Pyribenzamine and had proceeded to inject massive doses of the substance intravenously.

According to the military criminal investigator, who submitted the samples of the urine to the laboratory, "It had taken six men to subdue two of the prisoners who had gone completely berserk and the other three had gotten quite a 'bang' out of it." The quantity of the substance recovered in 100 to 200 ml. urine samples varied from 200 to 320 mg. per 100 ml.

Two other cases were studied in which the patients had believed they were "mainlining" heroin. In these two cases, the substances were Chlor-Trimeton and Thephorin, respectively.

The movements of the antihistaminics evaluated on the papers are very regular, and, if conditions are adhered to exactly, agreement of within ± 1 per cent on continuous runs is obtained with all the compounds shown in Table I. The effect of the pH 6.5 phosphate buffer, as can be seen from Fig. 5, is to increase markedly the movements of the antihistaminics and yet to limit the movements of most of the other alkaloids extracted. Whether or not this is due to the decreased solubility of the aqueous phase or due principally to the pH has not been thoroughly investigated.

Sensitivities of the various color reactions (Table I) were investigated. The CNBr benzidine reaction was sensitive from 40 to 50 μg per 4 mm. spot (method of Huebner[4]). The sensitivity of the fluorescence of the antihistaminics varied from 5 to 20 μg per 4 mm. spot. The other compounds examined showed slight flourescence in the 40 to 50 μg range but never with the intensity of those marked in Table I. The slight flourescence of the other compounds not marked as *F* in Table I completely disappeared on treatment with cyanogen bromide.

The ferricyanide-ferric chloride reaction was uniformly sensitive at 5 to 10 μg per 4 mm. spot.

Recoveries of the antihistaminics using the method for blood samples have been successful in all cases in which the substance was present in the urine. Due to the small amount of substance present in the blood, however, complete analysis could not be made. The chromatographic behavior of the urine-separated substances and those from blood were identical.

Recoveries in one case of liver tissue analysis showed the same substance as recovered from the blood and urine on subsequent chromatography (Pyribenzamine), and studies with guinea pig liver have given recoveries of 3 to 5 per cent of the injected substance. However, further work must be continued along this line. Recoveries from the crude Stass-Otto concentrates have been excellent, showing far more possibility in our hands than the classical method. Recoveries were in the order of 10 per cent on two Stass-Otto filtrates analyzed.

The three Rf's given for heroin were thought to be due to degradation products of the impure substance received from the Bureau of Narcotics. Due to the difficulty attendant on obtaining pure heroin, the impure product was used in determining the Rf's given in Table I.

Many other developing solvents, suggested by other workers, were investigated and some showed considerable promise.[6] Fifty per cent glacial acetic acid in isobutyl alcohol, developing Solvent 4, can be used, as results were quite consistent with this developing solvent (Fig. 3). However, the three solvent systems in Table I appeared to be quite sufficient for our work. Whatman No. 1 paper was investigated for use in this work but was found to be unsatisfactory due to the slow movement of the solvent front over the paper and the resultant poor spots and solvent fronts produced. The importance of the atmosphere solvent in the descending chromatogram jars cannot be overemphasized. The pair should be re-equilibrated after one passage of a chromatogram to insure uniformity of the atmosphere.

Ascending chromatography was investigated and was found to give quite satisfactory results. However, the apparatus was difficult to handle and the discarding of large volumes of developing solvent with each run was prohibitive.

We have successfully re-employed developing solvents in ascending chromatography. It is necessary, however, when employing this technique, to run standards simultaneously with the unknown, since Rf's calculated under these conditions are unreliable.

Ascending chromatography and comparisons of standards with unknowns given the authors as a test of the method have been completely successful and recommends itself highly to a small laboratory which does not run narcotics analyses as a routine part of its laboratory procedure. The method used was to roll three Whatman No. 4 (whole large sheets 57 by 47 cm.) papers into large cylinders, wash with 6.5 buffer and 1.00 N HCl, equilibrate for fourteen hours, lower into the appropriate developing solvent, and allow to advance to 25 cm. Dry, compare with standards, and treat as in the descending procedure, with the exception of the use of precalculated Rf values from Table I.

We chose 1.00 normal HCl saturated n-butyl alcohol because of the excellent movement and separation of the alkaloids it afforded, and because of the ease of preparation of the reagent.

Isoamyl alcohol gave approximately the same results on chromatography as did n-butyl alcohol and can be used as an alternate developing solvent exactly as outlined under the procedure.

Conditions of developing solvent must be maintained exactly as outlined, and chromatograms should be run in a small room set aside from the rest of the laboratory where extremes of temperature are not encountered. The room utilized in this laboratory for our work varied from 23° to 25° C., and with this variation of temperature significant changes in Rf values were not noted. In every instance in which an incorrect Rf for a given compound was obtained, the difficulty was traced to an improperly equilibrated atmosphere.

The use of the common alkaloidal color reagents, i.e., Marquis', Frohde's, Buckingham's, etc., for differentiation of the antihistaminics from the alkaloids was investigated. However, in many cases as with Trimeton and Thephorin, similar reactions were obtained. Dependence on these was therefore abandoned. These reactions can, however, be carried out on the chromatographed spots if so desired.

No systematic attempt was made to recover quantitatively the other alkaloids from the urine or blood as this has been adequately investigated by others. However, morphine was recovered in 8 cases in amounts varying from 120 to 550 mg. per 24 hours.

Differentiation of the compounds in pills and powders has been made rapidly and simply by this procedure. Ethylmorphine, codeine, and Demerol have all been identified in many cases, as well as mixtures of these compounds with the antihistaminics given in Table I.

Prevention of ion exchange causing spot abnormalities was realized by the use of an excess of electrolyte on the paper strips. This was accomplished by impregnating the papers with HCl and with pH 6.5 buffer (modification of Schute[5]).

Movements with buffers of varying pH were investigated. This work indicated that with some specific compounds greater separation than those obtained by the authors with the systems given could be obtained.[7] However, in an overall search for a large number of compounds in small quantities, the method given appeared to be superior.

SUMMARY

A method has been described for the determination of the antihistaminics which has been used successfully for a period of two years on a number of medicolegal cases.

It is felt that the use of minute quantities of the substances required for analysis and the multiplicity of tests, both physical and chemical, necessary to tie together a successful chemical investigation, makes this chromatographic technique an excellent one.

It also recommends itself because of the ease with which an inexperienced analyst can differentiate these compounds without the attendant confusion which results from exclusive reliance on the classical spot tests, or on ultraviolet absorption data.

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NONSPECIFIC FACTORS IN BLOOD COAGULATION

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RECENT studies of the blood-clotting mechanism have demonstrated that the conversion of soluble fibrinogen into insoluble fibrin is the result of a remarkably complex series of reactions involving many different enzymic moieties. Some of these moieties are presumed to undergo stoichiometric reactions to form active enzymes; others are described as accelerators, inhibitors, activators, or inactivators. Estimations of the concentration or activity of all of these elements are ultimately based on the time of clot formation in altered clotting systems specifically designed for the purpose. Indeed, the very discovery and description of each of the clotting factors depended upon observations on the delay or acceleration of clot formation in altered clotting systems.

It has been recognized for sometime that nonspecific salt factors affect the coagulation time of blood. Quick[1] stated that the mathematical equation expressing the relation between clotting time and thrombin required a corrective factor which depends upon concentration of electrolytes. Glazko and Greenberg[2] studied the inhibiting effect of various electrolytes and reported that polyvalent anions inhibited coagulation by acting directly on thrombin, whereas polyvalent cations acted upon fibrinogen to prolong clotting times. The relationship between coagulation time and NaCl concentration was investigated by Weitnauer, Grüning, and Wöhlisch.[3] They found a NaCl concentration of 0.5 per cent to be optimum for the complete clotting system. Astrup[4] pointed out that the effects of pH and ionic strength on the conversion of fibrinogen to fibrin was similar to their effects on other enzymic reactions. Loomis and Seegers[5] investigated the effects of salt concentration, temperature, pH and different ions upon the activation of purified prothrombin. They found 28° C. to be the optimum temperature at pH 7.2. Concentrations of $Mg(NO_3)_2$, NH_4Cl or NaCl above 0.9 per cent inhibited the activation of prothrombin. Ratnoff and Potts[6] found that calcium ions accelerated the conversion of fibrinogen to fibrin. They studied a number of other cations which accelerated the clotting of plasma by thrombin. The acceleration was not due to changes in ionic strength of the clotting system.

The nature and magnitude of nonspecific alterations must be understood in order that one may avoid the possible error of attributing the effects of differences in nonspecific components to specific clotting enzymes. Systems designed to estimate the "units per milliliter" of a given clotting component are particularly vulnerable to such error. Unless final concentrations of the reagents

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employed are consistently identical for each determination, the "units" of activity may not be comparable. At best, the "units" involved in such determinations are arbitrary. The preparation and use of relatively purified clotting reagents call for meticulous attention to the manner in which they influence the salt concentration, pH and protein content of the final reaction mixtures. Insufficient consideration of these factors may lead to serious artifacts in the estimation of clotting factors, and even to the proposal of the existence of accelerating or inhibiting factors which do not, in fact, exist. This paper presents data on nonspecific factors which are present in commonly used reagents and which may lead to false interpretation of clotting experiments.

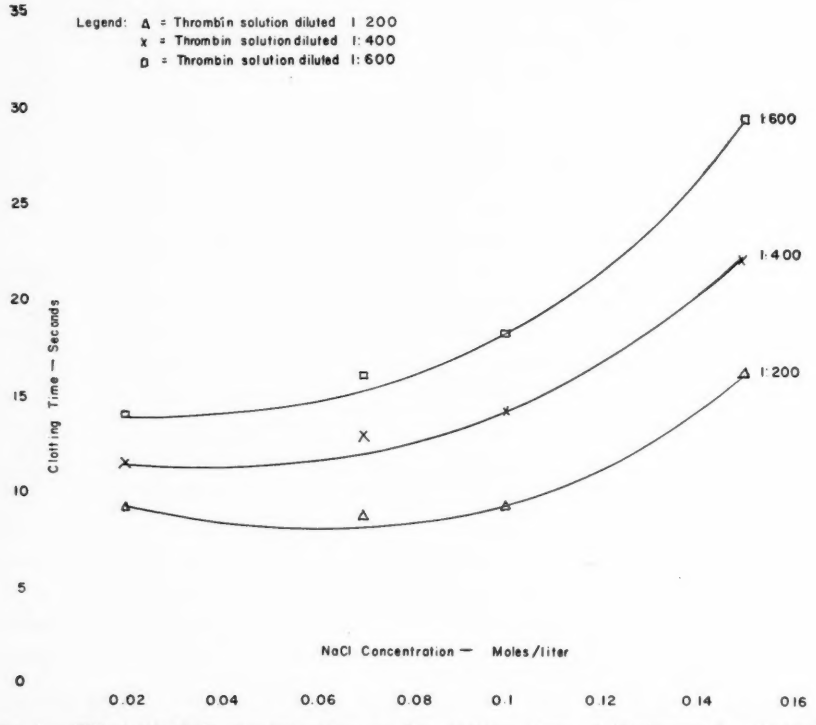

Fig. 1.—Effect of NaCl concentration on the clotting time of fibrinogen by thrombin.

DATA

A. Nonspecific influences on the fibrinogen-thrombin reaction:

1. In order to determine the effect of varying sodium chloride concentration on the rate of fibrinogen conversion by a standard thrombin preparation in the procedure used for the determination of thrombin units, the following experiment was performed:

Solutions of fibrinogen were prepared in 0.1 ml. aliquots containing 300 mg. fibrinogen* per milliliter. To each of the aliquots was added 0.4 ml. of freshly diluted thrombin solution (Parke-Davis Thrombin, topical) at 37° C., and the clotting times were noted. Saline solutions of varying concentrations were used as diluents of the thrombin. The final saline

*The fibrinogen employed was prepared by Warner-Chilcott Laboratories. It was free from citrate, thrombin, and prothrombin, and contained 0.85 per cent NaCl when reconstituted to contain 300 mg. clottable protein per milliliter.

concentrations are shown in the abscissa of Fig. 1. The influence of these different salt concentrations on three different thrombin concentrations was studied. From the figure it can be seen that it is meaningless to define a unit of thrombin as the amount capable of causing a clot in 15 seconds, unless the sodium chloride concentration of the final reaction mixture is specified.

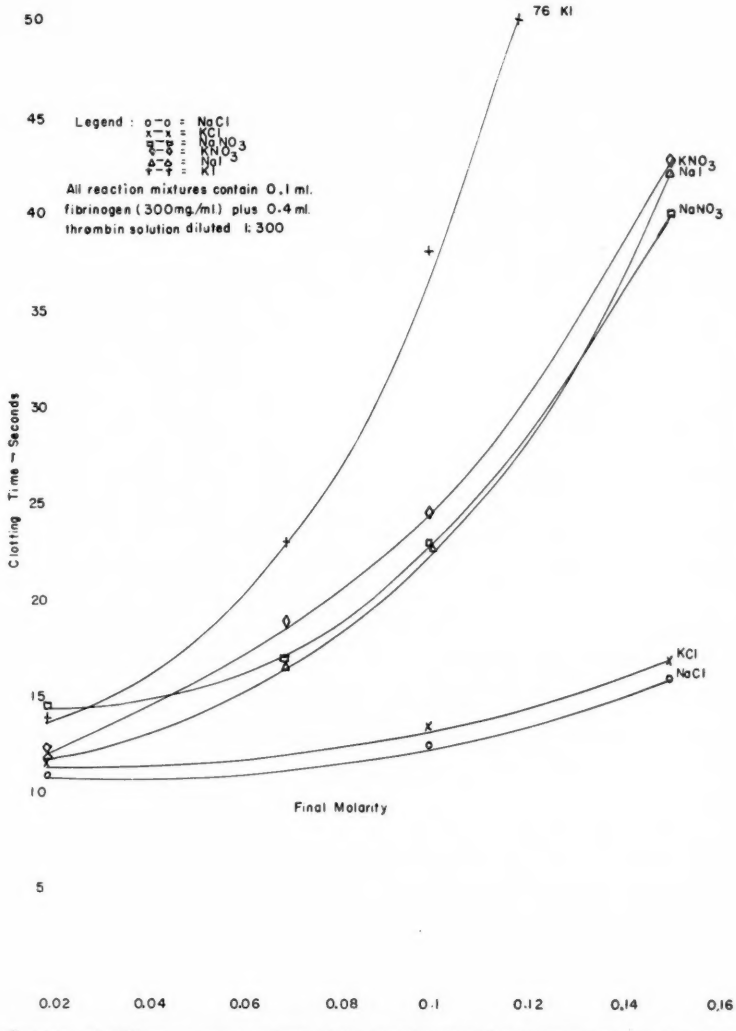

Fig. 2.—Influence of different monovalent salts at identical ionic strength on the clotting time of a standard thrombin-fibrinogen solution.

2. The influences of different ions at identical ionic strength were studied by taking identical aliquots as above, but using thrombin solution diluted 1:300 with different concentrations of NaCl, KCl, NaI, KI, $NaNO_3$ or KNO_3. Fig. 2 illustrates the results. Apparently the qualitative nature of the salt diluent may significantly influence the time of the clotting reaction.

3. The effect of fibrinogen concentration on the rate of conversion to fibrin by thrombin was also studied. The test aliquots contained identical (0.85 per cent) concentrations of sodium chloride and varied concentrations of fibrinogen, as shown in Fig. 3. This type of

activity-dilution curve is commonly observed in simple enzyme-substrate systems. It is clear that the "units of thrombin" found in any test solution will vary with the fibrinogen concentration of the final reaction mixture.

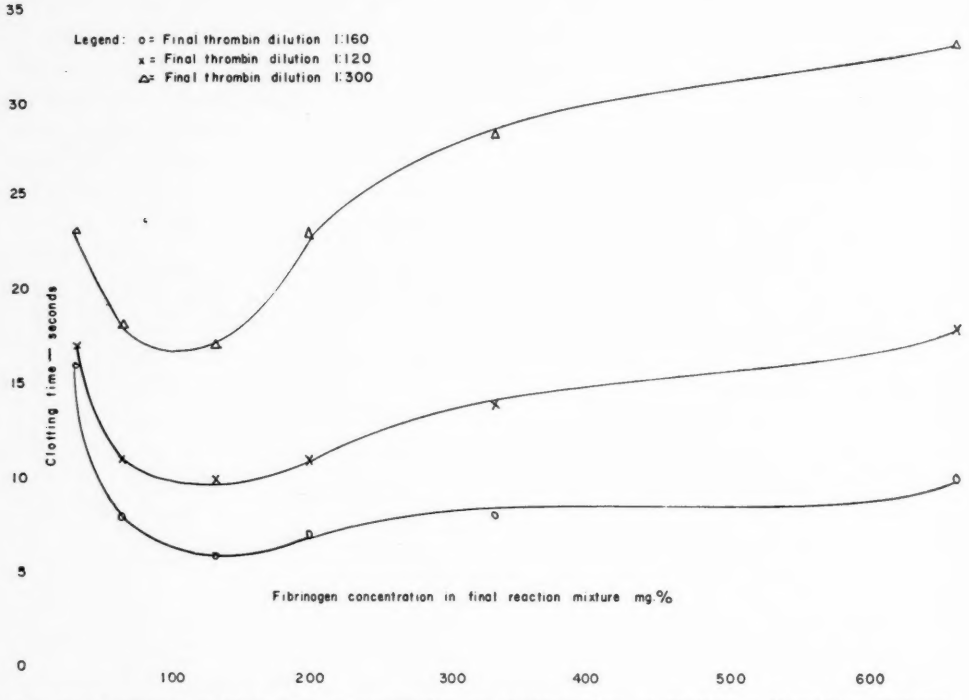

Fig. 3.—Influence of fibrinogen concentration on clotting time of fibrinogen-thrombin mixtures.

B. The two-stage prothrombin technique is based on the estimation of thrombin activity. Variations in final salt and protein concentrations may occur in the thromboplastin preparation or in the diluents used. The effects of such variations were investigated by the following experiments:

The two-stage test described by Ware and Seegers[7] was employed. Defibrinated plasma is serially diluted and the prothrombin in the diluted sample is converted quantitatively to thrombin by the addition of thromboplastin. Estimations of the thrombin activity in each dilution are then made at two-minute intervals. In each case a minimum reaction time (maximum thrombin activity) appears. That dilution which gives a minimum reaction time of 15 seconds is considered to contain one unit of prothrombin.

Parallel estimations were made with variously prepared thromboplastins using diluents of different salt and fibrinogen concentrations to see how these factors influence the apparent prothrombin content as determined by the two-stage method. Table I illustrates the variation introduced by changes in the salt concentration of the diluent. The manner in which the thromboplastin extract is prepared for use also influences the apparent content of prothrombin. This is illustrated in Table II.

C. The one-stage prothrombin estimation involves the use of fewer reagents and dilutions. Consequently there is less opportunity for nonspecific reagent properties to influence the reaction time. However, the salt concentration of the thromboplastin preparation must still be considered. Table III illustrates how the ionic strength of thromboplastin extracts may influence the reaction time. Since some of these extracts contain salt, activity may actually be reduced in the more concentrated thromboplastin preparations. This factor is

TABLE I. THE INFLUENCE OF VARIOUS SODIUM CHLORIDE CONCENTRATIONS IN THE TWO-STAGE PROTHROMBIN METHOD

NaCl CONCENTRATION OF FINAL REACTION MIXTURE		MINIMUM CLOTTING TIME OF FIBRINOGEN IN SECONDS
MG. %	MOLES/LITER	
68	0.012	22.0
150	0.026	13.0
240	0.041	12.6
300	0.051	11.5
350	0.060	12.4
400	0.068	12.8
600	0.103	18.5
850	0.145	31.0
1000	0.171	35.0

TABLE II. EFFECT OF ALTERING THROMBOPLASTIN PREPARATION ON THE APPARENT PROTHROMBIN CONTENT BY THE TWO-STAGE METHOD

PREPARATION OF THROMBOPLASTIN	MINIMAL CLOTTING TIME
Standard dilution (100%)	22.5
Diluted to 66% with water	16.5
Diluted to 33% with water	15.0
Diluted to 25% with water	12.5
Diluted to 17% with water	13.5
Diluted to 66% with 0.85% NaCl	23.0
Diluted to 33% with 0.85% NaCl	27.0

of considerable importance when the clotting activity of "concentrated" plasma* is determined. In preparing plasma for the diluted one-stage plasma prothrombin time, the influence of the salt concentration in the diluent is again demonstrated (Table IV). These

TABLE III. INFLUENCE OF SALT CONCENTRATION IN THROMBOPLASTIN PREPARATIONS ON THE ACCELERATED CLOTTING TIME OF PLASMA

FORM OF THROMBOPLASTIN	AS NORMALLY PREPARED		PREPARED DOUBLE CONCENTRATION*		DILUTED TO HALF CONCENTRATION WITH	
	METHOD	NORMAL TIME	METHOD	TIME	H_2O	SALINE
Simplastin, all reagents in one dry preparation	Dissolved in 4 ml. dist'd water	14 sec.	Dissolved in 2 ml. water	21 sec.	14 sec.	16 sec.
Difco desiccated rabbit brain	Extracted with 4 ml. 0.85% NaCl. $CaCl_2$ sol'n added later	14 sec.	Extracted with 2 ml. saline	14 sec.	15 sec.	17 sec.
Soluplastin solution of thromboplastin in saline	Mixed with equal vol. $CaCl_2$ sol'n	15 sec.	Dry $CaCl_2$ instead of $CaCl_2$ sol'n added	19 sec.	16 sec.	18 sec.

*The method of obtaining thromboplastin concentrations with these preparations results in a final mixture with about double the usual salt concentration and consequently with a prolonged clotting time in spite of increased thromboplastin concentration. The double concentration of Difco was prepared without increased salt concentration, and consequently with no rise in the clotting time.

same factors have a marked effect in the estimation of serum prothrombin activity (prothrombin consumption test).[8, 9] Since this test involves the simultaneous addition of a source of fibrinogen and a source of thromboplastin, the salt concentration of both reagents

*The one-stage prothrombin time of "whole" plasma is actually determined on a final reaction mixture containing about 25 per cent plasma. Some recent studies have been performed with preparations designed to measure the prothrombin activity in final reaction mixtures with up to 80 per cent plasma. Such mixtures have been found to have longer clotting times than the more diluted preparations.

TABLE IV. PROTHROMBIN TIMES OF PLASMA DILUTED EIGHTFOLD WITH SALT SOLUTIONS OF DIFFERENT CONCENTRATION

STANDARD THROMBOPLASTIN PREPARATION	% NaCl USED AS DILUENT			
	0	0.4	0.85	1.2
Simplastin 4802	29.5	31.5	35.0	43.0
Soluplastin 202641	36.5	37.5	40.0	49.5
Difco 411396	32.5	33.5	38.0	46.5

must be standardized. If, for example, the thromboplastin solution is used to dissolve the fibrinogen, the resulting combined reagent contains the sum of the salt contents of each. Such an undiluted combined reagent usually failed to clot when added to serum. Serial dilutions of the preparation with water gave increasingly short serum prothrombin times, leveling off at a normal minimum of about 25 to 30 seconds. When the combined reagent was similarly diluted with saline instead of water, the same sera failed to clot the reagent in less than 80 seconds.

Simple recalcification time may also be influenced by salt concentrations as shown by the following experiment:

The recalcification time of twelve normal plasma specimens was determined by three different techniques. The results are summarized in Table V. Comparison of the first two rows demonstrates the influence of saline.

TABLE V. INFLUENCE OF SALT CONCENTRATION ON RECALCIFICATION TIME

CONTENT OF FINAL REACTION MIXTURE			AVERAGE RECALCIFICATION TIME (SECONDS)
PLASMA (%)	MOLARITY OF ADDED SALINE	MOLARITY OF $CaCl_2$	
33	0	0.008	63
33	0.3	0.008	78
97	0	0.015	180

It is interesting to note that the system represented in the third row contained the highest concentration of plasma and, therefore, of all the clotting factors. Nevertheless, its clotting time was much slower than that of the more dilute preparations. However, since many other nonspecific factors are uncontrolled in the comparison, it would be hazardous to conclude that the longer clotting time of the concentrated reaction mixture is the result of a specific enzyme inhibitor.

DISCUSSION

Workers in the field of coagulation are generally aware of the influence of many nonspecific factors on clotting time. However, recent work defining several new specific clotting factors has resulted in a multitude of new techniques and reagents designed to measure these factors. Too often the techniques fail to consider the varying influences of the nonspecific factors in the diluents used. The data here presented confirm the importance of avoiding conclusions from comparisons of systems which are not identical in their concentrations of all nonspecific factors in the final reaction mixture.

The use of thromboplastin, fibrinogen, and altered plasma preparations calls for meticulous attention to the salt and fibrinogen concentrations of the final reaction mixtures. Differently prepared thromboplastins which give identical prothrombin times by a standard technique will not necessarily give identical results with altered plasmas, or when employed in a new technique. The use of fibrinogen preparations calls for attention to the ionic concentration. Fibrinogen containing citrate cannot be expected to yield the same results as citrate-

free fibrinogen, even if neutralized with excess calcium. Adsorption and elution experiments must be carefully standardized. A direct comparison of clotting times measured by two different techniques is of little significance.

SUMMARY

1. Nonspecific salt and protein concentration has been found to influence significantly the clotting mechanism as studied by recalcification time, one- and two-stage prothrombin time, and direct thrombin-fibrinogen conversion.

2. Different mono-monovalent salts in identical molecular concentrations may influence the clotting mechanism differently.

3. Systems measuring "units" of thrombin or prothrombin may yield significantly different results if carried out under conditions giving different salt or fibrinogen concentrations in the final reaction mixtures.

4. To avoid serious artifacts, thromboplastin preparations and other reagents used in clotting studies must be carefully standardized with regard to concentration of nonspecific components.

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A NEW TECHNIQUE FOR DROPPING THE CHORIOALLANTOIC MEMBRANE IN EMBRYONATED CHICKEN EGGS

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TECHNIQUES for dropping the chorioallantoic (CA) membrane of the embryonated egg vary widely. Many are time-consuming and several are unsatisfactory because nonspecific lesions frequently appear on the membrane after these procedures. In many laboratories the eggshell is penetrated with a high speed drill. Studies in this laboratory indicate that drilling per se regularly causes a lesion on the CA membrane beneath the drilled hole, presumably as a result of heat generated by the drill. After drilling through the eggshell, a second operation is required to puncture the eggshell membrane. This is also true when a simple punch is used of the kind now commercially available. Cutting windows or lifting shell flaps are even more time-consuming procedures.

To obtain dropped membranes free from damage, a new instrument has been designed for perforating the eggshell and the eggshell membrane. A simple and effective procedure for the use of this instrument has been devised.

As depicted in Fig. 1, *A*, the instrument consists of a curved brass bar acting as a handle, to which is attached a shell piercer with a wedge-shaped cutting edge. The instrument can be made readily. The following materials are needed: (1) a brass bar, 2½ inches long, ½ inch wide, and ⅛ inch thick; (2) a No. 18 nail 1½ inches long; and (3) a piece of brass rod ¼ inch in diameter. The brass bar is drilled at its center to receive the nail with a snug fit. The bar is bent to an arc to follow the lateral curvature of the egg. A hole is drilled through the brass rod, and the nail is inserted. The brass rod forms a collar around the nail and facilitates secure attachment of the nail to the bar. The brass rod and the nail are then soldered to the bar with the nail extending through the hole in the bar. At a length of 3/16 inch, the nail is clipped with a pair of wire-cutting pliers. This produces a chisel edge which is essential to the successful use of the instrument.

Due to the dimensions of the sharp wedge, the eggshell is penetrated with ease, and a sufficiently large opening is made for subsequent inoculation. Two factors are of importance to assure penetration of the eggshell membrane without puncture of the CA membrane: (1) After the wedge has passed through the eggshell and the eggshell membrane, but before the chorioallantoic membrane is punctured, the sense of resistance ceases, giving the operator an indication not to proceed further. (2) The danger of puncturing the chorioallantoic membrane is reduced because the piercer terminates in a cutting wedge rather than a needle point.

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The use of eggs not more than 12 days old is recommended because with older eggs bleeding frequently occurs when the membrane is dropped. The egg is candled and marked a short distance (0.5 to 1.00 cm.) from the air sac in an area free of large blood vessels. A drop of sterile saline is placed over the mark, and the piercer is applied to the eggshell through the drop of saline as shown in Fig. 1, *B*. It should be emphasized that if saline is not used, hemorrhagic lesions are produced regularly at the time of dropping the membrane.

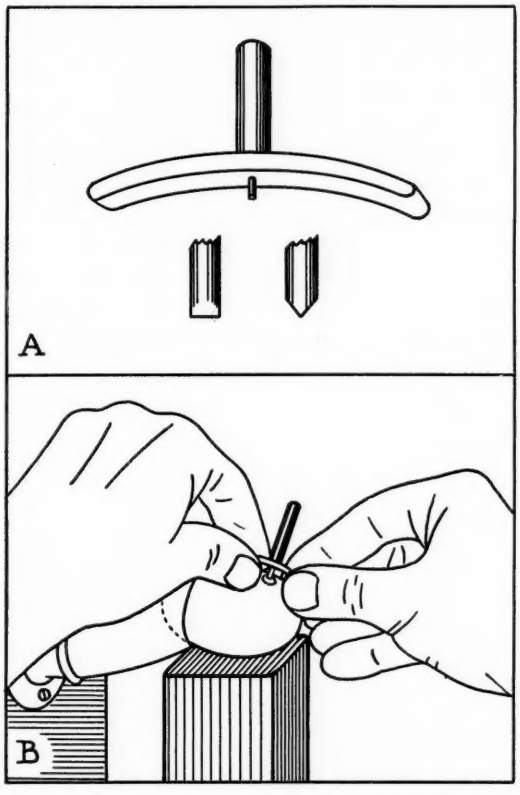

Fig. 1.—*A*, Instrument for perforating the eggshell and eggshell membrane. Enlarged views are shown of the cutting wedge. *B*, Perforation of the eggshell and eggshell membrane with the instrument. The egg rests on a support in front of a candler.

The piercer is pushed slowly through the eggshell and the eggshell membrane and along with it saline enters. When resistance ceases, the piercer is withdrawn. Saline spreads along both surfaces of the eggshell membrane. Holding the instrument by the rod, instead of by the bar, the piercer is now used to make an opening over the air sac. Suction is applied gently through this opening, and the CA membrane drops. A false air sac has been created and the original one obliterated. The hole over the false air sac is located eccentrically near the blunt end of the egg. To avoid back pressure and thus to assure unhindered and complete introduction of inocula with a volume of 0.2 ml. or greater, a second hole may be made over the false air sac. A small drop of mineral oil applied to the eggshell before this puncture is made prevents

eggshell material from dropping on the CA membrane. For inoculation a 25 gauge, 1½ inch needle is used. The needle is introduced to almost the entire length of the false air sac. The inoculum is injected stagewise as the needle is moved about and withdrawn. The eccentric hole makes distribution of the inoculum much easier. The shell holes are then sealed with paraffin.

Studies in this laboratory with the new technique have been concerned primarily with vaccinia virus, but the results obtained with this procedure should apply to any lesion-producing agent. The absence of traumatic damage during the process of dropping the membrane provides an ectodermal surface on which no nonspecific lesions are present which might interfere with the development and recognition of specific lesions.

The following representative example provides an indication of the precision of pock counts with vaccinia virus. Groups of 5 eggs were inoculated with a suspension of infected CA membrane diluted $10^{-6.75}$ with inactivated 5 per cent normal horse serum in buffered saline. Each egg received 0.2 ml. The dropped membranes were harvested 72 hours later and the pocks were counted. In the 8 groups inoculated the mean number of pocks per membrane in groups of 5 eggs ranged from 8.0 to 13.6. The grand mean was 11.2 with a standard deviation of 2.14. Considering each block of 4 groups separately, the means were 11.5 and 10.95, respectively, with the corresponding standard deviations, 2.28 and 2.29. A detailed report of quantitative studies with vaccinia virus is in preparation.

A FLASK FOR THE STUDY OF THE GROWTH DYNAMICS OF TUBERCLE BACILLI

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SOME of the potentialities of single and combined antibacterial drugs can be studied by observing the manner in which such drugs alter the normal growth curve of bacteria. The growth of bacteria dispersed in a liquid medium can conveniently be measured nephelometrically or turbidometrically when the cultures are growing in optically matched test tubes. This method, however, has certain drawbacks; a number of tubes must be used in each experiment to obtain an average rate of growth, and the small surface area of the culture medium limits the oxygen available to the organisms. In the case of tubercle bacilli cultured in Dubos liquid medium in test tubes the growth curve consists of a short lag phase, a short (2- to 3-day) logarithmic phase and then a prolonged arithmetic phase of growth. The change from a logarithmic to an arithmetic growth curve results from a limited oxygen supply, and the slope of the arithmetic portion of the curve is determined by the quantity of oxygen available to the culture. In some cases the limitations on growth, imposed by the small surface area of the culture, mark the effects of the antibacterial drug or combination of drugs under study.

To circumvent this difficulty a flask was designed which permitted a large surface area of medium. It also possessed a test tube side arm into which an aliquot of the medium could be tilted for nephelometric readings (see Fig. 1).

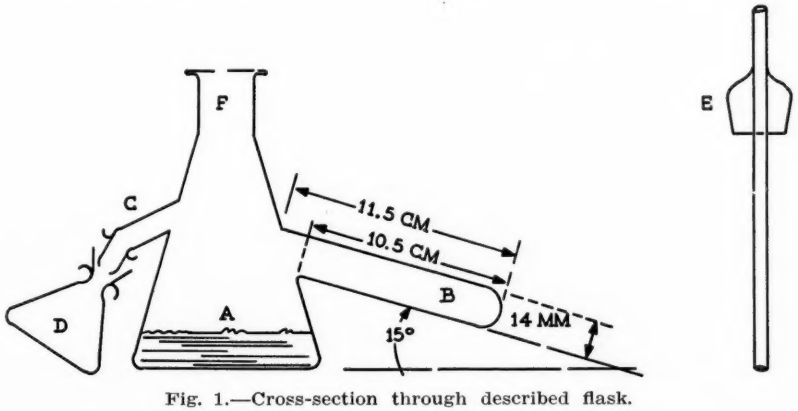

Fig. 1.—Cross-section through described flask.

A is a 500 ml. Erlenmyer flask; *B* is a 14 mm. test tube, 11 cm. long attached 5 cm. above bottom of flask *A* at a 15° angle to the horizontal; *C* consists of a side arm with a ground glass joint to fit the ground glass joint on a 25 ml.

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Erlenmyer flask *D*. This side arm may be used to tilt out a sample of the culture into flask *D* without danger of contaminating the main volume of medium. In our laboratory this arrangement is used to sample radioactive tubercle bacilli grown with C^{14} PAS, C^{14} INH, or C^{14} sodium acetate.

A ground glass joint *F* and tube *E* are optional but can be used to expose the culture to various concentrations of oxygen and CO_2. Otherwise the flask *A* can be stoppered with a sterile cotton plug.

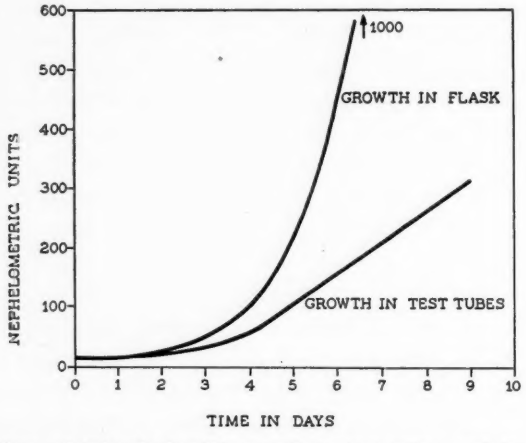

Fig. 2.—Comparison of tubercle bacilli growth in test tubes and in described flask.

Nephelometric readings are made by tilting an aliquot of the culture into the side arm *B* and placing *B* in the well of the nephelometer. Between readings the sample in *B* is tilted back again into flask *A*.

Fig. 2 compares the growth of tubercle bacilli strain H37Rv in test tubes with the growth in 25 ml. of Dubos medium in the flask described.

AN IMPROVED METHOD FOR THE DETERMINATION OF PLASMA HEPARIN

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IN A prior publication[1] the authors described a method for the determination of endogenous plasma or serum heparin utilizing an initial precipitation with N-octylamine, reprecipitation by zinc sulfate, splitting of the octylamine-heparin complex by treatment with basic methanol and acetone, and subsequent treatment to remove completely all traces of solvent. The final extract possessed anticoagulant, antithrombic, and metachromatic activity which was determined using a semimicro modification of The United States Pharmacopeia XIV anticoagulant method of assay for heparin sodium. Thus the existence of biologically active circulating heparin (s) in normal individuals was demonstrated. This has recently been confirmed in horse and man.[2, 3] However, we were unable to obtain more than a 45 per cent recovery of injected heparin using this method, and therefore suspected that the precipitation of endogenous heparin by octylamine was perhaps incomplete. Furthermore, octylamine is quite unstable and difficult to obtain and maintain in a pure form. For these reasons we have not been completely satisfied with it. Nevertheless, data obtained using the octylamine method[4] are valid for comparative purposes despite the incomplete heparin extraction.

Our previous studies had indicated that native circulating heparin is strongly protein bound. Furthermore, protamine neutralization of the anticoagulant activity of the final material was inconstant unless the extract was first subjected to tryptic digestion. These findings suggested the use of proteolytic enzymes to split the heparin-protein complex. The method described below is based upon the tryptic digestion of the total serum or plasma proteins previously precipitated by methanol-acetone with subsequent dialysis and lyophilization. The anticoagulant activity is measured as previously described.[1]

MATERIALS AND METHODS

The method requires no special reagents or equipment except for the trypsin which must be fairly pure. Crystalline trypsin is satisfactory but expensive. We have used a highly purified trypsin preparation (noncrystalline) in 50 per cent glycerol.* This material has a unit activity about one-half that of crystalline trypsin (salt free, Worthington).

Reagents and Materials.—

Methanol—C.P.

Acetone—C.P.

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*Purified Trypsin Concentrate (PTC) 50 to 100 mg. per milliliter in 50 per cent glycerol. Supplied by Southern California Gland Co., 4268 South Produce Plaza, Los Angeles, Calif.

Phosphate buffer 0.2 M, p.H. 8.5
Purified Trypsin Concentrate (PTC) solution (80 mg. per milliliter)
Dialysis tubing—¼" (Visking)

Procedure.—Place 5 ml. citrated plasma in a 25 ml. test tube; add 10 ml. methanol and 10 ml. acetone, shake vigorously, and allow to stand for at least one-half hour. Centrifuge and decant the supernatant. (Despite repeated tests using octylamine or methanol-acetone we have not found residual heparin activity in the supernatant.) Wash the precipitate twice with acetone using a glass rod to break up the precipitate thoroughly. Centrifuge and decant supernatant each time.

Thoroughly dry the coagulum under a vacuum aspirator. To prevent loss of the solid material due to rapid solvent removal, the stopper is covered with a fine cloth. While using the aspirator, the tube should be in a boiling water bath to facilitate drying. Add 5 ml. phosphate buffer (0.2 M, pH 8.5) and 0.5 ml. PTC solution to the dried precipitate, mix thoroughly, and allow to digest in a 37° C. water bath overnight. A highly viscid solution indicates that digestion was incomplete and fresh enzyme must be obtained. (Other proteolytic enzymes, papain and pepsin, singly and in combination, have also been tried but trypsin has been the most satisfactory.) When using PTC solution, 80 mg. per milliliter, less than 0.4 ml. has given incomplete digestion, whereas larger amounts than 0.6 ml. have resulted in decreased heparin activity.

Heat coagulate for one-half hour in a boiling bath. While boiling, it is desirable to use a fan on the upper portion of the test tubes, thus setting up a condenser. This prevents boiling over and evaporation. Cool, centrifuge, and decant and drain the clear supernatant into the dialysis tubing. (Repeated testing has failed to reveal heparin activity in the coagulum if the digestion has been complete.) Wash the funnel with approximately 1 ml. distilled water. Dialyze under running cold tap water overnight. Transfer the dialyzed solution into a 25 ml. test tube and lyophilize. Prolonged lyophilization (8 hours) may be necessary for thorough drying. Remove the dried sample, stopper, and store in refrigerator until ready to be tested.

The anticoagulant assay is performed as previously described.[1] However, since the final extract after dialysis is at neutrality, no adjustment of pH is necessary. The lyophilized material is dissolved in 1.0 ml. of isotonic saline or distilled water for the anticoagulant assay.

TABLE I. HEPARIN LEVELS (IN UNITS PER CENT) BEFORE AND AFTER OVERNIGHT DIALYSIS

	PLASMA NO. 1 WITH 0.4 ML. ENZYME	PLASMA NO. 1 WITH 0.5 ML. ENZYME	BLANK WITH 0.5 ML. ENZYME	COMMERCIAL HEPARIN 20 U. %
Dialyzed	17.5	17.5	0	20
Nondialyzed	30	38	10	19-20

TABLE II. RESULTS OF ANTICOAGULANT AND ANTITHROMBIC HEPARIN ASSAY (IN UNITS PER CENT) BEFORE AND AFTER OVERNIGHT DIALYSIS

	ANTICOAGULANT ASSAY	ANTITHROMBIC COFACTOR ASSAY*
Dialyzed	15-18	17.5
Nondialyzed	38-40	40-42

*Courtesy Dr. Gerald Lanchantin, Los Angeles County General Hospital.

RESULTS

Table I illustrates that dialysis is essential since some of the split protein products or denatured enzymes may interfere in the anticoagulant assay giving falsely high values. It also shows that commercial heparin does not dialyze using Visking dialysis tubing.

Table II demonstrates that dialysis is also essential if the antithrombic cofactor method is used for the final assay, and that the extract has equivalent

anticoagulant and antithrombic cofactor activity. Apparently the substance (s) which can give nonheparin anticoagulant activity also interferes with the antithrombic cofactor method of heparin assay.

TABLE III. EFFECT OF PROLONGED COLD DIALYSIS ON ANTICOAGULANT AND METACHROMATIC ACTIVITY

POOLED PLASMA	ANTICOAGULANT ASSAY	TOLUIDINE BLUE
Overnight dialysis	15 units %	No metachromasia
One-week cold dialysis	18 units %	Strong metachromasia

Table III shows that prolonged dialysis does not result in further decrease in the anticoagulant activity of the extract. Paper electrophoresis studies* have also confirmed that the anticoagulant activity is due to heparin. When undialyzed extract was used, a slow-moving ninhydrin staining spot was demonstrated, and also a faster-moving metachromatically staining spot on toluidine blue paper which was similar to commercial heparin. After three weeks of dialysis in the cold, only the heparin spot was present on paper electrophoresis. Table III also illustrates another curious finding. The extract obtained by the present proteolytic method contains a substance which inhibits the metachromatic activity of heparin on toluidine blue. In fact, when commercial heparin is added to a solution of toluidine blue which also contains the extract, no metachromasia occurs. However, this substance is removed by prolonged dialysis (one week). It is interesting to note that whereas it completely inhibited the metachromatic activity of heparin, it had slight or no effect upon the anticoagulant action.

Protamine sulfate causes a complete reversal of the anticoagulant action of the extract only after it has been subjected to prolonged dialysis. Reversal does occur when protamine is added to the overnight dialyzed extract, but the reaction is inconstant, perhaps because of the ninhydrin staining material found on paper electrophoresis.

TABLE IV. DUPLICATE ANALYSIS OF IN VIVO AND IN VITRO HEPARIN RECOVERY EXPERIMENTS. RESULTS IN UNITS PER CENT

	CONTROL		12′ AFTER 25 MG. HEPARIN I.V.		20 U. % HEPARIN ADDED TO PLASMA IN VITRO		% RECOVERY
Plasma No. 1	9	10	75	65			76
Plasma No. 2	13	12			28	30	82.5

Table IV illustrates the results of in vivo and in vitro recovery experiments analyzed in duplicate. Assuming 3 liters of plasma, there was 76 per cent recovery of injected heparin. In vitro recovery was 82.5 per cent. Using our previous octylamine method, in vivo recoveries varied from 40 to 45 per cent whereas in vitro recovery was negligible.

Table V presents duplicate determinations of the heparin content of 25 consecutive plasma samples. Thus the method in our hands has a ± 13 per cent deviation from the mean. Analysis of pooled plasma samples has given similar results.

*Performed at the Los Angeles County General Hospital through the courtesy of Dr. Gerald Lanchantin.

TABLE V. DUPLICATE HEPARIN ASSAY OF 25 CONSECUTIVE INDIVIDUAL PLASMA SAMPLES. VALUES IN UNITS PER CENT

CASE NO.	HEPARIN ASSAY NO. 1	HEPARIN ASSAY NO. 2	% DEVIATION FROM MEAN	CASE NO.	HEPARIN ASSAY NO. 1	HEPARIN ASSAY NO. 2	% DEVIATION FROM MEAN
1	18	16	6	14	13.5	13.5	0
2	15	15	0	15	13.5	13.5	0
3	17	14	10	16	12	13	4
4	12.5	13.5	4	17	13.5	14.5	4
5	15	17	7	18	9	10	5
6	12	12	0	19	12	13.5	6
7	15.5	14.5	3	20	12	12	0
8	17.5	20	7	21	10	13	13
9	17.5	17.5	0	22	17.5	19	4
10	17	17	0	23	20	20	0
11	20	18	5	24	13.5	13	2
12	16	18	6	25	14	14	0
13	17.5	17.5	0				

DISCUSSION

The results of in vivo recovery experiments with the new proteolytic method are superior to those previously obtained using octylamine. In view of the 15 to 20 per cent loss in the in vitro recoveries, and since a small part of the endogenous circulating heparin is adherent to the cells,[5] it is probable that a large percentage of circulating heparin (s) is assayed by the method outlined. This is substantiated by our inability, despite repeated extractions of various types, to detect heparin-like activity in the supernatant after the initial methanol-acetone precipitation, or in the coagulum formed after heat denaturation of the enzyme.

The results obtained again verify that heparin is normally present in the circulating blood, and that it is strongly protein bound. The nature of the method is such that the final material probably contains other mucopolysaccharides (as chondroitin sulfate) present in the plasma. However, since these do not possess substantial anticoagulant activity, they do not affect the assay of heparin(s).

SUMMARY

1. A new proteolytic method for the determination of plasma or serum heparin has been outlined.

2. The results of recovery experiments are superior to those obtained using octylamine, and indicate that the greater part (75 to 80 per cent) of endogenous circulating heparin is determined by this method.

3. In our hands the method has a ± 13 per cent deviation from the mean.

4. Evidence has been obtained that heparin is normally strongly protein bound.

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A MICRODIFFUSION METHOD FOR THE DETERMINATION OF PLASMA GLYCINE

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THE procedure for the quantitative estimation of glycine, described by Alexander, Landwehr, and Seligman,[1] and modified by Christensen and associates,[2] has been found, in this laboratory,[3] to provide requisite accuracy and sensitivity. However, the necessity for careful steam distillation of each of numerous samples made the procedure laborious and time-consuming. For this reason, the technique was adapted for use with a Conway microdiffusion unit,[4] a modification which has resulted in a considerable saving in applied time and effort with no loss in accuracy or sensitivity. With the technique presently reported, as many as 72 glycine determinations may be conveniently carried out simultaneously. This procedure has been successfully applied to the measurement of glycine in human and rat plasma or serum and rat diaphragm,[5] but, in the interest of brevity, only the plasma determination is described here.

MATERIALS AND METHODS

Reagents.—

1. Picric acid solution, 0.044 N. Twenty-two grams of picric acid are dissolved in 2,000 ml. of distilled water. This solution is titrated against 0.1 N NaOH using phenolphthalein as an indicator.

2. Ethyl phosphate (practical).

3. Chromotropic acid reagent.

STOCK SOLUTION: Five per cent (w/v) aqueous sodium chromotropate (sodium 1, 8-dihydroxynaphthalene-3, 6-disulfonate). This solution is stored in the freezer in a glass-stoppered brown bottle, thawed as needed, and then refrozen.

WORKING REAGENT: Six milliliters of the stock solution are diluted to 100 ml. with concentrated H_2SO_4. The volumetric flask is placed in cool water, and H_2SO_4 is added slowly with mixing to prevent overheating. Stored in a refrigerator in a glass-stoppered brown bottle, this solution remains stable for one week.

4. Citrate buffer, pH 5.0: Forty-two grams of citric acid are dissolved in 400 ml. of 1 N NaOH, diluted to 2,000 ml. with distilled water and then stored in the refrigerator.

5. Ninhydrin reagent: Two grams of ninhydrin are diluted to 100 ml. with citrate buffer. This solution is stored in a brown bottle. Fresh solutions are prepared weekly.

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6. Glycine standards:

STOCK SOLUTION containing 1 μM glycine per milliliter is prepared by dissolving 37.5 mg. glycine in 500 ml. of 0.044 N picric acid solution.

WORKING STANDARD contains 0.10 μM glycine per milliliter. Ten milliliters of the stock solution are diluted to 100 ml. with 0.044 N picric acid solution.

Apparatus.—

1. Conway diffusion vessels with appropriate glass covers.
2. Burette, 50 ml., amber-colored glass.
3. Matched test tube cuvettes.
4. Coleman Junior, Model 6A, spectrophotometer.
5. Metal racks for support of test tubes in a steam bath.
6. Racks for storing and manipulating Conway vessels. The safe and rapid handling of large numbers of Conway units is fraught with difficulties and discomfort for small-handed personnel. The use of simple, plywood racks, illustrated in Fig. 1, has greatly facilitated the efficient manipulation of these vessels.

Determination of Plasma Glycine Concentration.—One milliliter of heparinized plasma (or serum) contained in a 15 ml. centrifuge tube is diluted with 4 ml. of picric acid solution. The mixture is stirred, centrifuged, and glycine concentration of the clear supernatant solution is determined. Samples for analysis are run in duplicate. A 0.5 ml. or 1 ml. aliquot of the picric acid supernate is pipetted into the outer well of a Conway microdiffusion unit. The material should be placed near the outside rim of the outer well to prevent "creeping" into the center well during evaporation. Evaporation to dryness may be effected in approximately one hour by placing the units under an exhaust fan in a hood. Multiple Conway units are easily and rapidly handled and transported from desk top to hood for the evaporation using the large wooden racks of the type pictured in Fig. 1. To conserve space the side walls of these racks are constructed with grooves so that one may be firmly fitted on top of another.

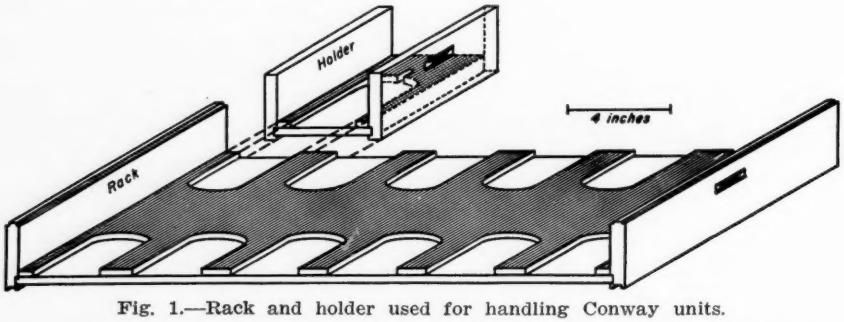

Fig. 1.—Rack and holder used for handling Conway units.

The rims of the Conway vessels are greased with a rather thick layer of petrolatum. This can be conveniently done with a syringe previously filled with melted petrolatum and cooled to room temperature. Five-tenths milliliter of ethyl phosphate is added to the outer chamber near the outside rim of each Conway vessel. A pipette with a rubber two-valve bulb should be used to avoid contact of this reagent with the skin or lungs. The unit is gently rotated so that the ethyl phosphate is uniformly distributed over the area previously covered by the picric acid solution.

The evaporation, greasing, and addition of ethyl phosphate is carried out serially on multiple Conway units. The next step, prior to storage in the dark, must be completed for each individual unit before proceeding to the next unit because of the light sensitivity of the chromotropic acid reagent. We have found that mixing of the contents of inner and outer wells is easily avoided during handling if units are placed in an individual wooden holder (Fig. 1). Using a 50 ml. amber-colored burette, 2 ml. of the chromotropic

acid reagent is added to the center well. Next, 0.5 ml. of the ninhydrin reagent is placed in the outer well. A glass cover is quickly placed over the greased rim with gentle tapping to expel air bubbles left in the petrolatum seal. An airtight seal is, of course, essential. To insure uniform mixing of the ninhydrin reagent, the unit is rotated slowly, with care, so that the chromotropic acid does not spill out of the inner well. The unit is transferred to the large wooden rack, without jarring by sliding the unit forward by means of a pencil placed in the small groove in the wooden holder. The units are permitted to stand overnight in the dark while the formaldehyde distills over into the center well.

On the following morning, the covers of the Conway vessels are removed and, prior to transfer to 13 by 100 mm. test tubes, the contents of the inner well are stirred gently with the pipette tip. The test tubes, protected from direct light, are heated in a boiling water bath for thirty minutes. After cooling, the solutions are transferred to calibrated test tube cuvettes and read at 570 mμ in the spectrophotometer. A glycine standard containing 0.10 μM glycine per milliliter and a reagent blank consisting of 1 ml. of 0.044 N picric acid are run in duplicate through the entire procedure concurrently with the unknown. The reagent acid blanks are read against a distilled water blank and the standard and unknowns are read against a reagent blank. "Creeping" of picric acid from the outer into the inner well will produce a pink color in the inner well after overnight standing. Such blanks or samples give fallaciously high readings and must be discarded.

RESULTS

Standard Curve.—A standard curve, illustrated in Fig. 2, has been found to follow Beer's law for concentration of glycine at least as high as 0.12 μM per milliliter. Calculations of glycine content are based on the glycine standard run concurrently with the samples to be analyzed.

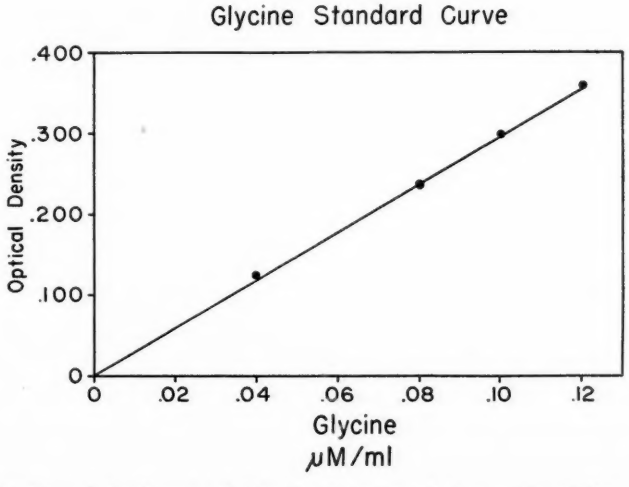

Fig. 2.—Demonstration of the linear relationship between glycine concentration and color yield.

Reproducibility and Recovery of Glycine.—A representative experiment illustrating the reproducibility of the procedure as well as the recovery of glycine added to multiple aliquots of a sample of normal human plasma is shown in Table I. In each instance, the quantity of glycine recorded, dissolved in 0.5 ml. of 0.85 NaCl, was added to 1 ml. aliquots of the plasma. After stirring, the mixture was brought to a volume of 5 ml. with picric acid solution. The tech-

nique outlined previously was then followed to provide the results indicated. The lower recovery values noted following the addition of 1 μM of glycine are explicable in terms of deviation from the linear relationship of optical density to glycine concentration at high color intensities.

TABLE I. REPRODUCIBILITY AND RECOVERY IN THE DETERMINATION OF PLASMA GLYCINE

SAMPLE		GLYCINE ADDED (μM)	OPTICAL DENSITY	GLYCINE FOUND (μM/ML.)	PER CENT RECOVERY
Reagent Blank*		0	0.068 0.062		
Standard†		0.10	0.222 0.228		
Normal Human Plasma† (aliquots of a single sample)	1	0	0.095 0.097	0.212	
	2	0	0.094 0.104	0.225	
	3	0	0.095 0.097	0.212	
	4	0.25	0.209 0.212	0.465	99.6
	5	0.25	0.207 0.207	0.458	96.8
	6	0.50	0.318 0.318	0.704	97.6
	7	0.50	0.325 0.320	0.712	99.2
	8	0.75	0.430 0.434	0.956	98.7
	9	0.75	0.440 0.438	0.971	100.7
	10	1.00	0.510 0.510	1.128	91.2‡
	11	1.00	0.520 0.520	1.151	93.5‡

*Read against distilled water.

†Read against reagent blank.

‡These decreases in percentile recovery result from a departure from Beer's law at higher optical density values.

Effect of pH and Salt Concentration.—Maximum color is obtained at a pH of approximately 5.0. The 0.1 M citrate buffer used in the ninhydrin reagent, creates the proper conditions for color development with regard to pH and salt concentration. With unbuffered picric acid (pH around 2.0) very little color is developed and the color produced does not conform with Beer's law. The use of a more concentrated (0.2 M) citrate buffer in the ninhydrin reagent results in a considerable decrease in color yield.

Effect of Time.—Distillation of formaldehyde into the inner well of the Conway unit is completed after approximately five hours of standing, although usually the purple color does not appear until after heating in a boiling water bath. We have found the procedure to be more adaptable to the laboratory day (particularly where a large number of analyses is required) if the units are incubated overnight or approximately sixteen hours.

Optical density readings were found to be essentially unchanged forty hours after development of the color.

SUMMARY

A relatively simple, accurate, specific, and highly reproducible method for the determination of glycine has been presented.

As little as 0.02 μM of glycine can be determined by this procedure. The application of this method to the estimation of glycine in picric acid supernates of human plasma is described. Recoveries of 98.7 ± 1.4 per cent are obtained when glycine is added to normal human plasma.

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A MICROMETHOD FOR DETERMINATION OF SERUM CALCIUM

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SCHWARZENBACH and associates,[1] reported that calcium in solution could be measured by direct titration of the sample at pH 11.5 to 12 with a standardized solution of a chelating agent, disodium ethylenediaminetetracetic acid, with ammonium purpurate as an indicator. This principle has been applied to determination of calcium in serum.[2, 3] The poor visual end point when this titration was tried on samples of blood serum led to modifications in which the end point was determined spectrophotometrically.[4, 5] Sobel and Hanok[6] reported the direct titration of serum calcium with disodium ethylenediaminetetracetic acid with eriochrome black T as the internal indicator in a solution buffered at pH 9.5. Under these conditions the titration measures total calcium plus magnesium. The end point is also a difficult one, particularly if the serum is pigmented. We have found that the principle of this titrimetric method can be satisfactorily applied to the determination of calcium in 0.2 ml. of serum if the calcium is precipitated as the oxalate, the calcium oxalate then dissolved in a measured excess of disodium ethylenediaminetetracetic acid buffered at pH 9.5, and the excess of the chelating agent measured by back titration with standard $CaCl_2$ solution. The presence of a limited amount of oxalate does not interfere with the titration so that the precipitate does not have to be washed. The excess of precipitating reagent and most of the serum magnesium is removed by aspiration of the supernatant fluid after centrifugalization of the precipitate. The elimination of washing removes one of the objections to micromethods involving precipitation of calcium as the oxalate.

The calcium is precipitated at pH 4.5 to 5.0 with mechanical agitation so that precipitation is virtually complete at the end of 30 minutes. If necessary, the determination can be finished within 1 to 2 hours after the serum sample is obtained so that the delay due to the precipitation step is not great. Eriochrome black T can be used as the indicator since the traces of magnesium in the small amount of supernatant fluid left with the precipitate can be neglected. The visual end point is fairly sharp as the serum pigments are removed. Only one accurately standardized solution is required, the $CaCl_2$ solution.

SOLUTIONS

Oxalate Reagent.—Five-tenths milliliter of 0.1 N oxalic acid is added to 10 ml. of 0.1 N sodium oxalate.

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Disodium Dihydrogen Ethylenediaminetetracetic acid (EDTA).—A stock solution of 4.5 Gm. of the salt is dissolved in distilled water and made up to 1 L. A working solution of 2 ml. of stock solution is diluted to 10 ml. with 0.25 M ethanolamine. This is freshly prepared each day.

Buffer Solution.—0.25 M ethanolamine–15 ml. ethanolamine is diluted to 1 L with distilled water.

(Stable if refrigerated.)

Indicator.—Stock solution: (a) Eriochrome black T solution*—Fisher Indicator for water hardness by Schwarzenbach, or (b) 0.5 per cent W/V solution of eriochrome black T powder, National Aniline, in ethanolamine.

Working solution: add 0.5 ml. of stock indicator *a* to 2.0 ml. of 0.25 M ethanolamine, or 0.5 ml. of stock indicator Solution *b* to 2.0 ml. of distilled water. The working solutions must be freshly prepared before each series of determinations.

Standard $CaCl_2$ solution: 0.02 M $CaCl_2$ prepared from $CaCO_3$ – 1 ml. = 0.8 mg. Ca.

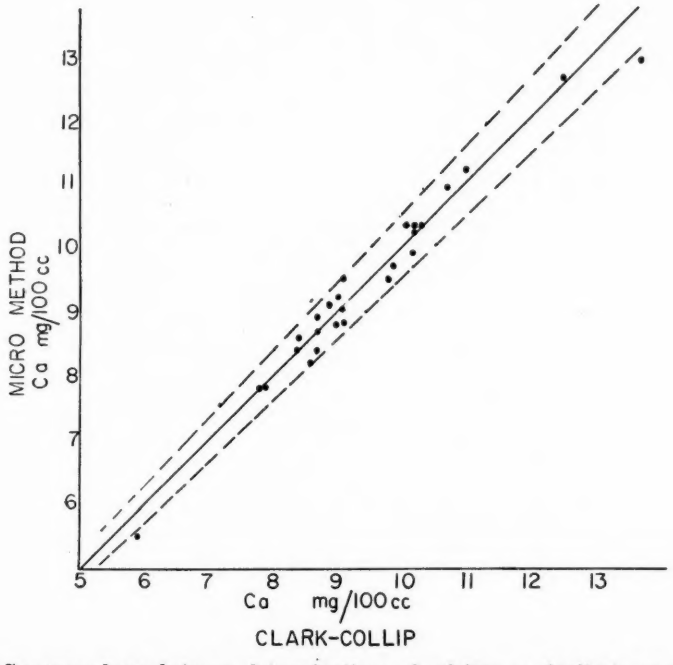

Fig. 1.—Correspondence between determinations of calcium on duplicate samples of serum by micromethod and by macromethod (Clark-Collip modification of Kramer-Tisdall method[7]). Dotted lines enclose area of ± 5 per cent agreement between the two determinations.

PROCEDURE

Two-tenths milliliter of serum is pipetted into a conical 2-ml. centrifuge tube† and 0.2 ml. of oxalate reagent added; the tubes are placed in a rack and shaken for 30 minutes in a shaker (Kahn test type). Initially, the tubes were allowed to stand for 2 to 3 hours after shaking but the precipitation is practically complete at the end of the shaking period, so that this is not necessary. Duplicate determinations which were carried through immediately after the shaking period have checked satisfactorily with those in which the tubes were allowed to stand several hours after shaking. The tubes are centrifuged for 5 minutes

*Certain batches of Eriochrome black T solution, Fisher, gave less distinct end points than others. It was found that the end point became sharper if the solution was shaken with anhydrous sodium sulfate and filtered. The solution was then stable if refrigerated and the diluted indicator prepared from these treated stock solutions gave satisfactory end points.

†Micro-calcium tube, Sobel, obtained from Emil Greiner Co., New York, N. Y.

and the supernatant fluid is removed by aspiration without disturbing the precipitate. Exactly 0.5 ml. of the diluted EDTA solution in ethanolamine buffer is added and the tubes tapped gently until the precipitate is loosened and completely dissolved. A 0.05 ml. portion of the working indicator solution is added and the solution is titrated with the standard $CaCl_2$ reagent using a microburette with a capacity of 0.1 ml.* The tip of the microburette is submerged in the solution and stirring is accomplished by bubbling air into the solution through a piece of fine polyethylene tubing extending to the bottom of the tube. A good titration illuminator is necessary. At the end point the indicator color changes from a blue-green to a purple-red. A blank titration in duplicate is made each time with 0.5 ml. portions of the same EDTA solution used for the unknown.

Calculations:

B = blank titration in milliliters.
U = titration of unknown in milliliters.

$$(B\text{-}U) \times 0.8 \times \frac{100}{0.2} = \text{mg. Ca/100 ml.}$$

Fig. 1 shows the results of duplicate determinations made simultaneously on serum samples by the Clark-Collip modification of the Kramer-Tisdall method[7] and the micromethod described. Each point represents a single serum sample. The correspondence between the two methods is good. The dotted lines which enclose almost all of the points indicate the limits of ± 5 per cent from theoretical agreement.

SUMMARY

A micromethod for the determination of calcium in serum is described requiring only 0.2 ml. of serum. The method gives results which are in good agreement with those obtained by the Clark-Collip method. The determination can be completed within 1 to 2 hours after the serum sample is obtained.

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*A Gilmont micro-burette, 0.1 ml. capacity, has been satisfactory.